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Orientadores: Prof. Doutor João Manuel Braz Gonçalves

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Tese especialmente elaborada para obtenção do grau de Doutor em Farmácia, especialidade Biotecnologia Farmacêutica.

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Perdigão P, Gaj T, Barbas III C, Santa-Marta M, Gonçalves J. Reactivation of latent HIV-1 expression by engineered TALE transcription factors. ESGCT and FSGT Collaborative Congress 2015, Helsinki, Finland.

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ABSTRACT

Despite the success of antiretroviral therapy, a cure for HIV-1 infection remains elusive. The persistence of cellular reservoirs harboring transcriptionally silent (latent) HIV provirus is responsible for the viremia rebound observed following treatment withdrawal. Stimulation of latent viral expression is considered critical to target HIV reservoirs for elimination through a "shock and kill" approach. Pharmacological drugs have systematically proven ineffective to drastically reduce the reservoir size and may cause severe side effects owing to their indiscriminate mode of action. In the present thesis, gene-targeted strategies were explored to stimulate and eliminate HIV latent cells. To stimulate latent virus expression, we designed synthetic activators based on transcription activator-like effector (TALE) proteins that recognize conserved regions on HIV 5'LTR promoter. Four TALE activators strongly induced HIV transcription, acting in cooperation to specifically enhance viral expression from cell line models of HIV-1 latency. Moreover, we show that histone deacetylase inhibitors can further enhance the effect of TALE-mediated activation in highly repressed latent cells. To further potentiate the elimination of stimulated latent cells, we conjugated an HIV-responsive suicide lentivector to our TALE activator technology. For this purpose, we incorporated a modified 5'LTR promoter into the suicidal lentivector as a safety mechanism to dissociate TALE-driven activation, restricting the responsiveness of this plasmid to the HIV regulatory proteins. The therapeutic plasmid was capable of specifically eliminate latently infected cells stimulated by TALE activators through a Tat/Rev-dependent expression of the diphtheria toxin. Finally, we presented a "gene-free" approach to specifically activate latent HIV expression through protein delivery of cell-penetrating zinc-finger activators (CPP-ZFA). A single activator based on Cys2His2 zinc-finger domains proved effective at inducing viral expression from the primer binding site downstream of 5'LTR promoter. When conjugated with positively charged nuclear localization signal repeats, this synthetic activator efficiently translocated across cell membrane without the need of carriers. Short-term presence of CPP-ZFA following protein delivery was sufficient to stimulate gene expression in HIV-1 latent cells, offering a safer alternative to avoid off-target effects from prolonged exposure to these synthetic activators. In resume, this work provides proof-of-concept that synthetic activators and suicide lentivectors constitute promising candidates for the eradication of HIV-1 reservoirs through gene-targeted strategies.

Key-Words: Human Immunodeficiency Virus type 1 (HIV-1); Latency; Gene therapy; Synthetic activators; Suicide lentivectors.

RESUMO

A Síndrome de Imunodeficiência Adquirida (SIDA) constitui uma das principais preocupações de saúde pública a nível mundial, sendo detetados anualmente mais de 2 milhões de novos casos. Esta doença é caracterizada por uma deterioração progressiva do sistema imunitário do hospedeiro que culmina na ocorrência de infeções oportunistas fatais. Desde a sua descoberta em 1983, o agente etiológico da SIDA, o Vírus da Imunodeficiência Humana (VIH), tem sido um dos patógenos mais investigados no desenvolvimento de estratégias terapêuticas. O VIH é um lentivírus pertencente à família *Retroviridae*, tendo sido até agora identificados dois tipos: VIH-1 e VIH-2. O VIH-1 é mais patogénico do que o seu homólogo do tipo 2, sendo caracterizado por uma taxa de transmissão elevada e maioritariamente responsável pela pandemia global de SIDA. Como tal, a infeção pelo VIH-1 tem sido o foco no desenvolvimento de estratégias antirretrovirais.

Apesar dos constantes avanços no desenvolvimento de fármacos antirretrovirais, estes não são capazes de eliminar completamente o vírus do organismo. O maior obstáculo a esta erradicação é a existência de reservatórios virais persistentes. Estes reservatórios são constituídos por células infetadas que não são eliminadas do hospedeiro, principalmente devido à capacidade do VIH-1 de estabelecer um estado não-replicativo (latente). Esta latência viral é caracterizada como um estado de infeção não expressivo que permite a evasão do vírus à resposta do sistema imunitário e à atividade farmacológica do tratamento antirretroviral. Como tal, estes reservatórios contribuem para uma viremia residual, e asseguram o restabelecimento da infeção ao ocorrer uma interrupção na terapêutica. Por outro lado, os principais reservatórios de VIH-1, os linfócitos T $CD4^+$ de memória, persistem nos pacientes devido ao seu tempo de meia-vida prolongado, evidenciando a importância da eliminação destes reservatórios virais.

Devido à sua natureza silenciosa, que permite a evasão dos reservatórios virais, é considerado essencial a estimulação do VIH latente de modo a expor estes reservatórios ao efeito tóxico da replicação viral ou à resposta do sistema imunitário do hospedeiro. Entre as diversas estratégias propostas para eliminar estes reservatórios, os fármacos que causam uma estimulação de vias de sinalização celular ou alteração de marcadores epigenéticos têm sido dos mais testados como ativadores da expressão do VIH latente. No entanto, estas abordagens não foram capazes de reduzir significativamente os reservatórios virais em pacientes. Por outro lado, a ação generalizada destes fármacos tem sido associada a efeitos colaterais geralmente causados pela desregulação do sistema imunitário. Um tratamento eficaz contra os reservatórios

de VIH-1 deve ter como base o desenvolvimento de uma terapia que seja capaz de eliminar especificamente as células infetadas, sem causar danos a células saudáveis. Deste modo o desenvolvimento de terapias alternativas, tal como a terapia génica, mostra especial interesse.

A terapia génica consiste na transferência de material genético exógeno para células-alvo, promovendo a alteração do seu fenótipo. Até ao momento vários tipos de abordagens antirretrovirais tendo como base esta terapia foram testadas principalmente com o objetivo de potenciar o sistema imunitário, mediar a morte de células infetadas ou tornar as células do hospedeiro resistentes à infeção. O objetivo proposto na presente dissertação é focado no desenvolvimento de estratégias terapêutica que permitam eliminar especificamente células infetadas com VIH-1 latente através de engenharia de ativadores sintéticos e vetores lentivirais suicidas. Uma área da terapia génica com particular relevância é a engenharia do genoma através de plataformas de ligação ao ADN. Estas proteínas podem ser manipuladas para reconhecer sequências específicas de ADN genómico e causar modificações na sua sequência ou alterar o nível de expressão génica, dependendo da função que lhes é atribuída através da ligação a um domínio funcional. Nesse sentido, a engenharia de ativadores sintéticos poderá ser uma abordagem importante no contexto de latência do VIH. Estes fatores de transcrição artificiais são formados por um domínio de ligação ao ADN que reconhece uma sequência predeterminada e um domínio de ativação que recruta a maquinaria celular para promover a transcrição do gene-alvo.

No Capítulo II descrevemos o desenvolvimento de ativadores sintéticos com base em proteínas TALE (do inglês *Transcription activator-like effector*) com o objetivo de induzir a expressão de VIH em células latentes. Estas plataformas de ligação ao ADN com origem nas bactérias *Xanthomonas sp* são maioritariamente constituídas por módulos de 34 resíduos conservados, cuja composição determina a afinidade para o tipo de ácido nucleico. Como tal, a junção contígua destes domínios numa ordem específica determina a sequência-alvo de ADN. Ativadores TALE foram desenhados em fusão com o domínio ativador VP64 para reconhecer sequências conservadas na região promotora do VIH, denominada 5'LTR. Quatro ativadores TALE foram selecionados como potentes estimuladores da expressão viral num modelo celular de latência. Determinados contextos de elevada repressão viral estão frequentemente associados com marcadores epigenéticos tais como a desacetilação de histonas que podem limitar o acesso de fatores de transcrição à região promotora do VIH. Como tal, demonstrámos que inibidores de desacetilases de histonas podem atuar em sinergia com os ativadores TALE aumentando

drasticamente a capacidade destes para reativar a expressão viral em contextos adversos. Deste modo, os ativadores TALE apresentam-se como uma abordagem promissora e segura para a estimulação de reservatórios virais.

Neste capítulo, explorámos ainda o uso de vetores lentivirais suicidas com o objetivo de promover especificamente a eliminação das células latentes estimuladas pelos ativadores TALE. Esta abordagem é baseada na construção de um plasmídeo lentiviral que codifica uma toxina letal, cuja expressão é dependente da presença das proteínas virais Tat e Rev. Como base para as construções, utilizámos um vetor lentiviral cuja transcrição é controlada pelo promotor 5'LTR ativado pelo transativador Tat e que possui ainda 2 locais de *splicing* do VIH entre os quais foram clonados os genes das toxinas e um local de ligação da proteína Rev (RRE). Este vetor tem a particularidade que, ao iniciar a sua transcrição intensificada pela presença de Tat, o RNA mensageiro incorporando a toxina só é transportado para o citoplasma para tradução na presença de Rev, impedindo a sua expressão células em não infetadas. Uma vez que pretendemos atacar células latentes, na qual a expressão de Tat e Rev é inexistente, este plasmídeo tem de atuar em conjunto com os ativadores TALE gerados. Estes ativarão a expressão viral, forçando a saída de latência e expondo a célula latente à ação da toxina e consequente morte celular.

De modo a conjugar este plasmídeo com ativadores TALE, modificámos o seu promotor 5'LTR – idêntico ao promotor nativo do VIH-1 – de modo a evitar ligação das proteínas TALE e ativação inespecífica em células não infetadas. A incorporação do promotor modificado reduziu drasticamente a expressão do vetor lentiviral na presença dos ativadores TALE, conferindo maior especificidade à ação mediada pelas proteínas virais Tat e Rev. Por outro lado, esta modificação no vetor não alterou a capacidade infeciosa dos lentivírus, que demonstraram elevada expressão apenas em células infetadas. Este efeito manteve-se mesmo quando testados como lentivírus não-integrativos que são frequentemente implementados em abordagens de terapia génica, onde se pretende evitar efeitos adversos da integração no genoma da célula. Para a morte específica de células VIH latentes, recorremos à elevada potência da toxina da difteria que promove diretamente a morte celular através da inibição da síntese proteica. Ao testarmos o vetor lentiviral suicida em conjunto com ativadores TALE, verificámos elevada morte de células infetadas com VIH latente em comparação com células não infetadas. Face à incapacidade do sistema imunitário para combater células infetadas, esta abordagem poderá potenciar a eliminação de reservatórios virais após a sua estimulação e como

tal, apresenta-se como uma nova estratégia de terapia genética para combater os reservatórios de VIH-1 latente.

No capítulo III testámos uma estratégia alternativa de ativação da expressão de VIH latente sem recorrer à introdução de material genético ao explorar a capacidade intrínseca de proteínas de dedos-de-zinco para penetrar na membrana celular. Estas proteínas humanas podem ser manipuladas para reconhecer sequências específicas de ADN de acordo com a composição de aminoácidos presente na sua hélice-alfa, que determina a afinidade para três ácidos nucleicos ou tripletos. A elevada frequência de resíduos de aminoácidos com carga positiva na sua estrutura promove a interação e internalização destas proteínas através da membrana lipídica aniónica, conjugando a função de reconhecimento de ADN e translocação celular na mesma molécula. Entre diversos ativadores dedos-de-zinco desenvolvidos para reconhecer o promotor 5'LTR em fusão com o domínio ativador VP64, apenas um foi capaz de induzir expressão viral em células latentes, ligando-se a uma região altamente conservada a jusante do promotor do VIH-1. Ao aumentar a sua carga positiva através da conjugação com péptidos sinalizadores catiónicos, este ativador consegue penetrar diretamente nas células VIH latentes e estimular a expressão viral, demonstrando a sua versatilidade como molécula de transporte celular e manipulação da expressão génica. Observámos que esta proteína persiste na célula durante apenas 48 horas, o que, no entanto, é suficiente para estimular a expressão de VIH latente. Esta abordagem é particularmente relevante na redução de potenciais efeitos adversos noutros genes derivados da exposição prolongada à ação dos ativadores sintéticos no ambiente intracelular.

Em resumo, os resultados obtidos nesta dissertação de doutoramento demonstram o potencial terapêutico de ativadores sintéticos e vetores lentivirais para combater os reservatórios de VIH latente, abrindo caminho para uma nova geração de moléculas terapêuticas para o tratamento da infeção pelo VIH.

Palavras-chave: Vírus da imunodeficiência humana (VIH-1); Latência; Terapia génica; ativadores sintéticos; vetores lentivirais suicidas.

ABBREVIATIONS

AAV	Adeno-associated virus
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ADA-SCID	Adenosine deaminase-deficiency SCID
ADN	Ácido desoxirribonucleíco
AdV	Adenoviral vector
AIDS	Acquired immunodeficiency syndrome
ART	Antiretroviral therapy
AS	Acceptor splicing site
aza-CdR	5-aza-2'deoxycytidine
Brec1	Broad HIV-recombinase 1
C/EBP	CCAAT-enhancer-binding protein
CA	Capsid
CAR	Chimeric antigen receptor
Cas9	CRISPR associated protein 9
CCR5	CC Chemokine Receptor 5
CD3	Cluster differentiation 3
CD34	Cluster differentiation 34
CD4	Cluster differentiation 4
CDK9	Cyclin-dependent kinase 9
cDNA	Complementary DNA
CMR-1	Chromosome region maintenance *1*
CMV	Cytomegalovirus promoter
CNS	Central nervous system
CPP	Cell-penetrating peptide
CPP-ZFA	Cell-penetrating zinc-finger activators
Cre	Cyclization recombinase
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRM-1	Chromosome region maintenance 1
CTD	C-terminal domain
CTL	Cytotoxic T lymphocyte
CXCR4	CXC Chemokine Receptor 4
CycT1	Cyclin T1
DBD	DNA-binding domains
dCas9	deactivated Cas9
DNA	Deoxyribonucleic Acid
DNMT3a	DNA (cytosine-5)-methyltransferase 3A
DPBS	Dulbecco's phosphate buffer solution
DS	Donor splicing site
DSB	Double-strand breaks
dsDNA	Deoxyribonucleic Acid
DSIF	DRB Sensitivity Inducing Factor
DTA	Diphtheria toxin
E2C	E2-Crimson
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
EF-2	Human elongation factor 2
ELISA	Enzyme-linked immunosorbent assay
Env	Envelope protein
FBS	Fetal bovin serum
LTR	Long-terminal repeat
LV	Lentiviral vector
MA	Matrix
MFI	Mean fluorescence intensity
miRNA	microRNA
mLTR	mutant LTR
mRNA	Messenger RNA
NC	Nucleocapsid
Nef	HIV negative factor
NELF	Negative elongation factor
NES	Nuclear export sequence
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor kappa B
NHEJ	Non-homologous end joining
NLS	Nuclear localization sequence
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
PAM	Protospacer adjacent motif
PBMC	Peripheral blood mononuclear cells
PBS	Primer-binding site
PHA	Phytohemagglutinin
PKC	Protein kinase C
PMA	Phorbolmyristate acetate
Pol	Polymerase
PolII	RNA Polymerase II
PR	Protease
P-TEFb	Positive transcription elongation factors b
rAAV	Recombinant adeno-associated virus
Ran	Ras-related nuclear protein
Rev	Regulator of Virion Protein
RNA	Ribonucleic Acid
RNAi	RNA interference
RRE	Rev-responsive element
RT	Reverse Transcriptase
RTA	Ricin toxin A
RV	Retroviral vectors
RVD	Repeat-variable diresidues
S/MAR	Scaffold/matrix attachment region
SAHA	Suberanilohydroxamic acid
SAM	Synergistic Activation Mediator
SCID	Severe combined immunodeficiency
SCID-X1	X-linked SCID
SDF1	Stromal cell-derived factor 1
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIDA	Síndrome da Imunodeficiência Adquirida
SIN-LTR	Self-inactivating LTR
siRNA	small interfering RNA
SLO	Streptolysin O
Sp1	Specificity protein 1

Fluc	Firefly luciferase	**SSC**	Side scatter
FSC	Forward scatter	**SV40**	Simian virus 40
Gag	Group-specific-antigen protein	**TALE**	Transcription activator-like effector
GDP	Guanosine diphosphate	**TALEN**	Transcription activator-like effector nuclease
GFP	Green fluorescent protein	**TAR**	Trans-activating response element
gp120	Glycoprotein 120	**Tat**	Trans-activator of Transcription
gp41	Glycoprotein 41	**TBP**	TATA-binding protein
gRNA	guide RNA	**TCR**	T-cell receptor
GTP	Guanosine triphosphate	**TET**	Ten eleven translocation
HA	Hemagglutinin	**TFIIB**	Transcription factor II B
HAART	Highly active antiretroviral therapy	**TGD**	Thymine DNA glycosylase
HDAC	Histone deacetylase	**TNF-α**	Tumor necrosis factor-alpha
HDR	Homology-directed repair	**TRIM5cyp**	Tripartite motif-containing protein 5 cyclophilin A
HEK	Human embryonic kidney	**TRIM5α**	Tripartite motif-containing protein 5 alpha
HIV	Human Immunodeficiency Virus	**Ubi**	Ubiquitin promoter
HIV-1	Human Immunodeficiency Virus type 1	**VEGF**	Vascular endothelial growth factor
HIV-2	Human Immunodeficiency Virus type 2	**Vif**	Viral Infectivity Factor
HLA	Human leukocyte antigen	**VIH**	Vírus da Imunodeficiência Humana
HSC	Hematopoietic stem cell	**VIH-1**	Vírus da Imunodeficiência Humana do tipo 1
IDLV	Integration-deficient lentiviral vector	**VIH-2**	Vírus da Imunodeficiência Humana do tipo 2
IL	Interleukin	**VP16**	Virus protein 16
IL-10	Interleukin-10	**VP64**	Virus protein 64
IL-2	Interleukin-2	**Vpr**	Viral protein R
IL-6	Interleukin-6	**VPR**	VP64-RTA-p65 tripartite fusion
IL-7	Interleukin-7	**Vpu**	Viral protein U
IN	Integrase	**VSV-G**	Vesicular stomatitis virus G protein
iPSC	induced pluripotent stem cells	**WPRE**	Woodchuck Hepatitis Virus Post-Transcriptional Regulatory Element
IPTG	Isopropyl-β-D-thiogalactopyranoside	**ZF**	Zinc-finger
IRES	Internal rybossome entry site	**ZF**	Zinc-fingers
KRAB	Krüppel-associated box	**ZFA**	Zinc-finger activator
LDL	Low-Density Lipoprotein	**ZFA**	Zinc-finger activator
LEGDF	Lens epithelium-derived growth factor	**ZFN**	Zinc-finger nuclease
LFN	Lethal factor N-terminal	**β-gal**	β-galactosidase
LoxP	Locus of X-over P1	**γ-RV**	Gamma-retroviral vectors
LSD1	Lysine-specific histone demethylase 1		

Amino acids

A - Alanine **G -** Glycine **M -** Methionine **S -** Serine **C -** Cysteine **H -** Histidine **N -** Asparagine **T -** Threonine **D –** Aspartic acid **I -** Isoleucine **P -** Proline **Y –** Tyrosine **E –** Glutamic acid **K -** Lysine **Q -** Glutamine **V -** Valine **F -** Phenylalanine **L -** Leucine **R -** Arginine **W –** Tryptophan

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CHAPTER I

General Introduction

1.1. Human immunodeficiency virus

The Human Immunodeficiency Virus (HIV) was discovered in 1983 as the causative agent of the acquired immunodeficiency syndrome (AIDS) [1]. HIV infection remains as one of the most predominant epidemics worldwide, with approximately 37 million people currently living with HIV, leading to over 1 million deaths per year caused by AIDS (Fig. 1.1; World Health Organization Database, 2016). AIDS is characterized by the suppression of the immune system in otherwise healthy individuals, leading to the emergence of opportunistic diseases that eventually leads to the death of the HIV infected patients [2–4]. Two types of HIV were identified so far: HIV-1 and HIV-2. Main concerns have been focused on HIV-1 as its transmission is responsible for AIDS global pandemic, being highly pathogenic. Due to its lower capacity of transmission, HIV-2 has been mainly restricted to the region of West Africa [5,6].

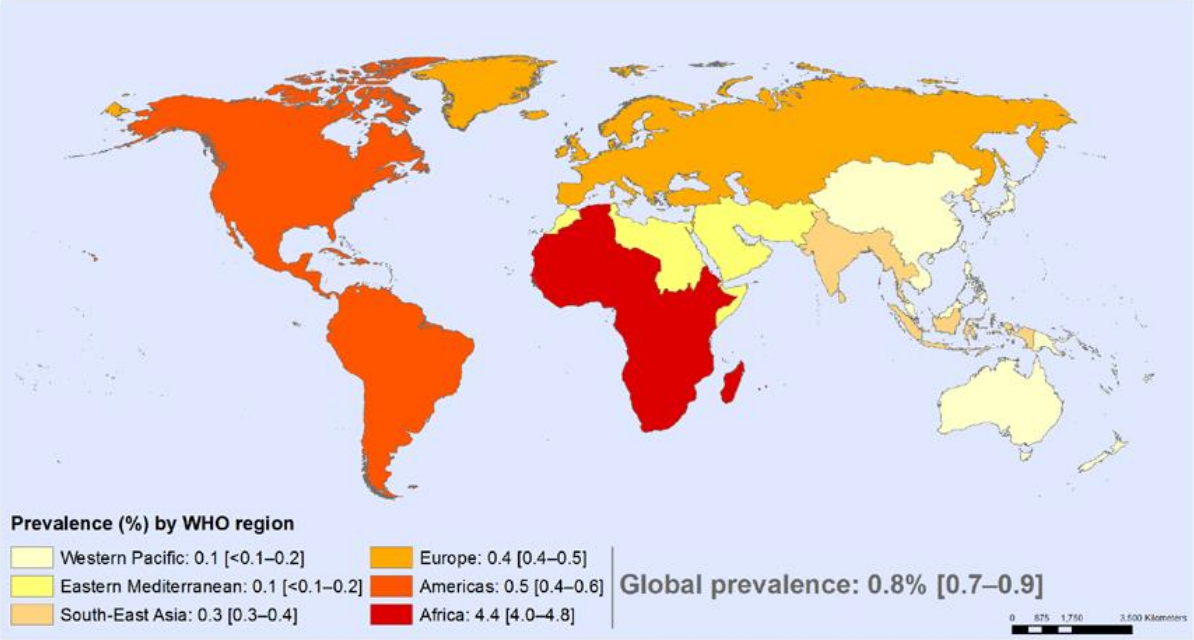


Figure 1.1- Global HIV adult prevalence (15-49 years) by 2016. 36.7 million [34.0–39.8 million] people were living with HIV at the end of 2015. An estimated 0.8% [0.7-0.9%] of adults aged 15–49 years worldwide are living with HIV, although the burden of the epidemic continues to vary considerably between countries and regions. Sub-Saharan Africa remains most severely affected, with nearly 1 in every 25 adults (4.4%) living with HIV and accounting for nearly 70% of the people living with HIV worldwide. Source: World Health Organization, 2016.

The main targets of HIV are CD4+ T lymphocytes and macrophages [7–9]. Briefly, when it enters the host, HIV directly infects these cells in the blood or mucosa. Spread of infection in lymphoid tissues leads to the viremia observed during acute phase of infection. The viremia

is controlled by the host immune response that mostly contains viral spread and the patient enters a phase of clinical latency. However, HIV continues to replicate leading to a gradual decline in CD4+ T cells and sequentially the patient develops clinical symptoms of AIDS [10]. The central nervous system (CNS) is also affected by HIV-1 invasion, particularly through infected macrophages that cross the blood-brain barrier and migrate to the brain. Infection of resident macrophages and microgial cells in the CNS can lead to neurodegenerative symptoms derived from inflammatory response to infection [11].

HIV tropism is mostly determined by the presence of cell surface receptor Cluster Designation 4 (CD4) and co-receptor CC Chemokine Receptor 5 (CCR5) or CXC Chemokine Receptor 4 (CXCR4) that mediates binding and entrance of the virus into target cells (Figure 2) [12]. HIV viruses that bind CCR5 (R5-tropic) co-receptors are the most commonly transmitted viral strains, being predominant during early stage of infection. CXCR4 targeted (X4-tropic) HIV strains tend to emerge later during disease progression to AIDS [13,14]. Dual-tropic strains (X4R5-tropic) capable of targeting both co-receptors emerge during transition from CCR5 to CXCR4 tropism.

1.1.1. HIV-1 genome and structure

The Human Immunodeficiency Virus belongs to the *Retroviridae* family as a member of the Lentivirus group. The HIV virus particle packages two copies of the viral RNA genome. Retroviruses are characterized by the presence of a Reverse Transcriptase (RT) and an Integrase (IN) that are essential to the viral replication cycle. The RT enzyme reverse-transcribes their positive genomic RNA into a proviral DNA, while the Integrase promotes the integration of the viral DNA into the host cell genome [15]. HIV-1 genome consists in a 9.8 Kb single-stranded positive RNA molecule, flanked by terminal repeats that are converted to 5’ and 3’ long-terminal repeats (LTRs) after RNA is reversely transcribed into cDNA and integrated in the host cell genome. This integrated form of HIV is denominated a provirus. The HIV-1 promoter controlling virus transcription and expression is comprised within the 5’ LTR region. HIV-1 genome encodes for 9 open reading frames (Fig. 1.2). The *gag*, *pol* and *env* genes encode for polyproteins which are then lysed by the viral Protease (PR) into individual proteins: the *gag* (group-specific antigen) region codes for structural proteins – MA (matrix), CA (capsid), NC (nucleocapsid), and p6; *pol* (polymerase) region encodes for essential enzymes participating in the HIV replication cycle – Protease (PR), Reverse Transcriptase (RT) and Integrase (IN); and *env* (envelope) region encode glycoproteins present in the outer

membrane of the virion that mediate binding and entrance into host cells – SU surface/gp120 (SU) and transmembranar/gp41 (TM) [16].

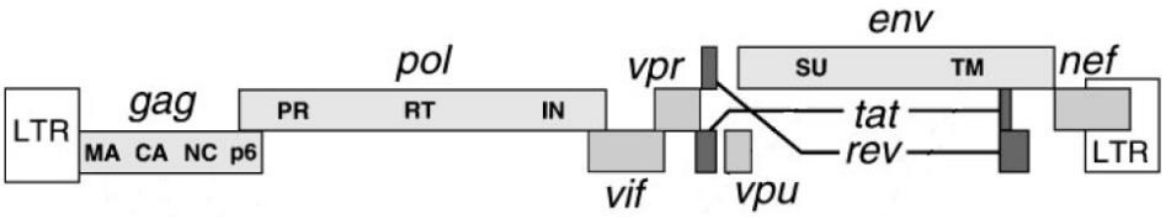
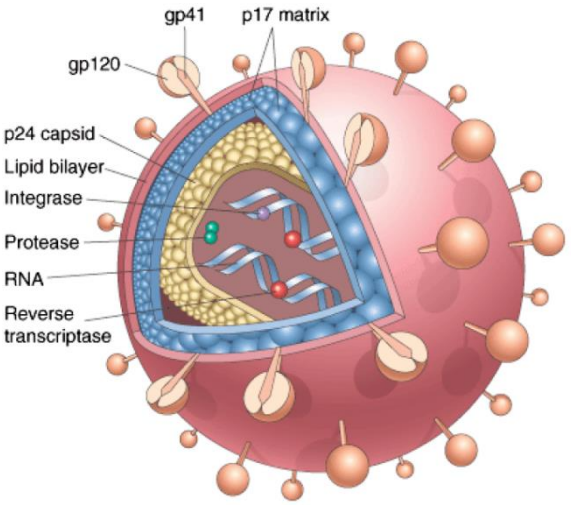


Figure 1.2- Organization of the HIV-1 genome. Source: *Frankel and Young 1998* [16].



Accessory genes (*vpr*, *vif* and *nef*) and regulatory genes (*tat*, *rev*, *vpu*) are also encoded by HIV-1 genome, presenting a particular role in HIV-1 entry and replication. Regulatory Tat, Rev and Vpu proteins are not incorporated in the HIV-1 mature virion (Fig. 1.3).

Figure 1.3- Structure of mature HIV-1. HIV-1 virion consists in a capsid particle, incorporating 2 RNA genome copies, along with reverse-transcriptase, integrase and protease enzymes. Accessory proteins Vif, Nef and Vpr are also packaged in viral particles. Matrix proteins stabilize capsid, interacting with viral envelope, a bilayer lipid membrane containing surface glycoproteins gp120 anchored to the capsid by the transmembranar protein gp41 Source: *Kumar et al. 2010* [17].

1.1.2. HIV-1 replication

HIV-1 replication cycle can be divided into 2 distinct stages: early (infection) and late (expression) phase (Fig. 1.4). Early phase of HIV-1 replication consists in the binding and entry steps of the mature virion into the cell. Binding occurs by specific interactions between the viral glycoprotein gp120 and the amino-terminal immunoglobulin domain of the cellular CD4 molecule, exposing the CCR5 or CXCR4 co-receptors [18,19]. As mentioned above, these co-receptors determine HIV-1 tropism [20]. The interaction of gp120 with these co-receptors causes conformational changes in the transmembrane protein gp41 that, in turn, exposes an N-terminal hydrophobic region ("fusion peptide"). Exposure of this region mediates fusion of viral and cellular membranes, and the subsequent entry of the virion core into the cell cytosol [18,19,21].

Once inside the cell, capsid proteins are dissociated from the viral core, which is converted to a reverse-transcription complex by a process called "uncoating". In this complex, the RNA genome remains associated with the nucleocapsid, matrix, reverse-transcriptase, integrase and the accessory protein Vpr. Following uncoating, the single-stranded RNA(+) genome is converted in the cytosol to a double-stranded linear DNA molecule by the reverse-transcriptase. Reverse-transcription of the RNA genome is initiated after the binding of a cellular $tRNA^{Lys}$ primer [15,18,19]. The newly formed cDNA molecule is transported to the nucleus as part of the pre-integrative complex containing the RT, IN, MA and Vpr proteins. This nuclear import is assisted by Vpr, which connects the complex to the nuclear import machinery [22]. The viral cDNA is then integrated into the host cell chromosome by the viral integrase, thus concluding the early phase of infection [18,19].

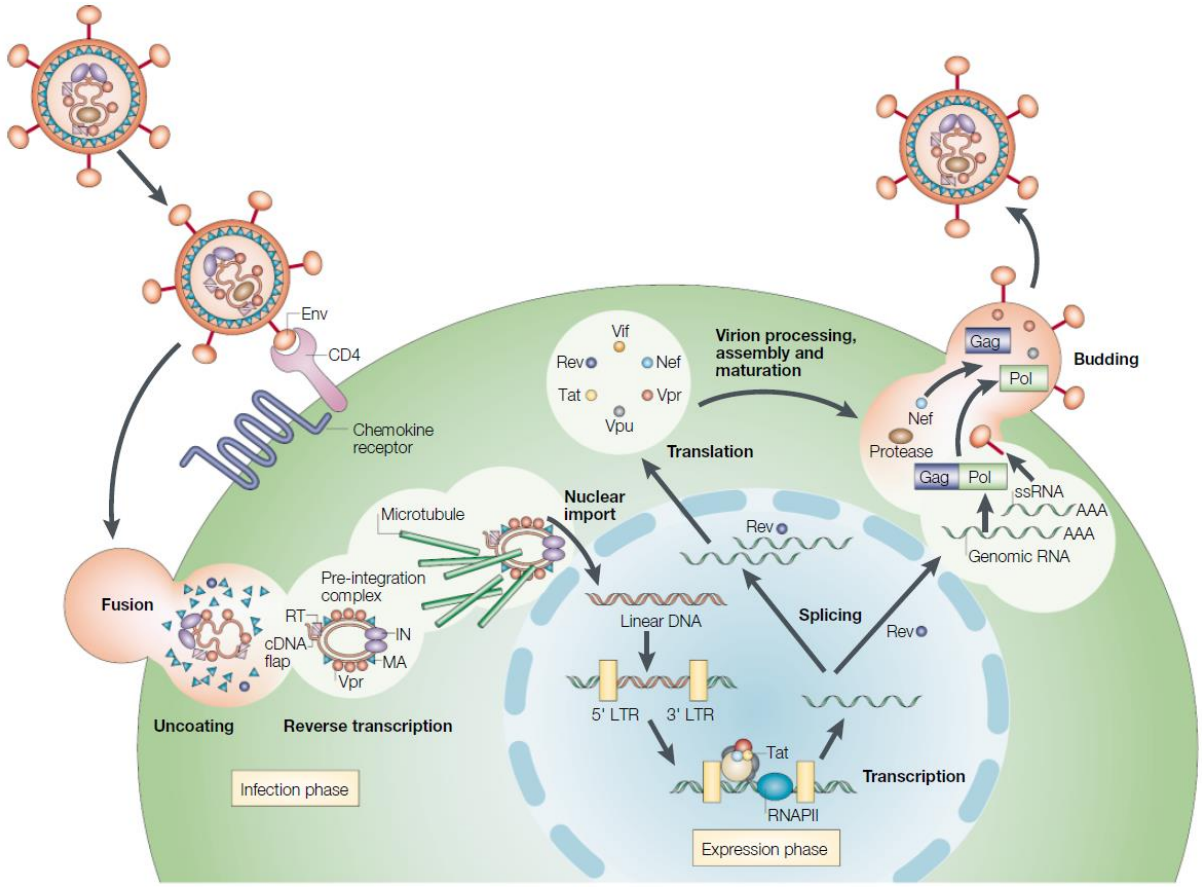


Figure 1.4- HIV-1 replication cycle. HIV-1 particles bind to target cell through interaction between viral envelope (Env) gp120 and cell CD4 receptor and a chemokine co-receptor. Viral and cellular membranes are fused and the virion core is released into the cell cytosol. In the cytosol, single-stranded RNA genome is converted to a double-stranded cDNA molecule by Reverse-transcriptase. Viral cDNA is imported into the nucleus as part of a pre-integration complex and integrated into host cell chromosome by viral Integrase. Host RNA polymerase II (RNAPII) promotes transcription of integrated proviral genome. Regulatory Tat assists transcription of elongated transcripts while Rev mediates exportation of single-spliced and unspliced transcripts to the cytoplasm.

HIV-1 full-transcripts are assembled with Gag and Pol polyproteins near the cell membrane and incorporated into immature virions. Viral particles are released from the cell by budding with cellular membrane. Viral protease cleaves GagPol polyprotein into individual functional proteins, triggering the maturation of virions by promoting the reorganization of their core and enabling them to infect new cells. Source: *Peterlin et al. 2003* [23].

The late phase of infection begins with the transcription of the provirus by the host RNA Polymerase II. At first, only Tat, Rev and Nef are initially expressed by means of alternative multiple splicing of the HIV-1 transcript. Tat has an essential role in this stage, activating the 5' LTR promoter and thereby enhancing the rate of HIV-1 transcription and elongation. Rev regulates transcription exportation by promoting export of single-spliced and unspliced RNA to the cell cytosol. Once Rev is expressed, single-spliced RNAs (*env*, *vif*, *vpu* and *vpr*) and unspliced RNAs (*gag* and *gagpol*) are translated in the cytoplasm [18]. More details on the Tat and Rev function are described below in section 1.1.2.1.

Gag and GagPol polyproteins are transported to the cell membrane where assembly of new viral particles occurs. Envelope proteins gp120 and gp41 are directed to the membrane through the ER-Golgi pathway. HIV-1 full-transcript RNA is encapsulated and immature virions are released from the cell. The viral protease triggers subsequently the maturation of virions, promoting the reorganization of their core and enabling them to infect new cells [18].

1.1.2.1. Tat and Rev regulatory function in HIV-1 replication

Several accessory proteins contribute to the efficiency of HIV-1 infection, replication and spread. After provirus genome integration into host chromatin, expression of Tat and Rev is a critical step in HIV-1 replication. In the absence of these viral proteins, the replication cycle is stalled, preventing efficient production of viral particles [18].

Host RNA polymerase II is capable of successfully initiate provirus transcription from 5' LTR HIV-1 promoter which contains binding sites for several transcription factors, such as NF-κB, Sp1 and TBP and contributes to the basal transcriptional activity of the viral promoter. However, RNA pol.II-mediated transcription fails to perform elongation of the RNA chains. The transcriptional transactivator Tat is the actual responsible for the bulk of viral transcription in more than two orders of magnitude above basal transcription [16,18]. Tat binds the trans-activating response element (TAR) stem-loop located at 5' end of the nascent RNA chain and recruits host positive transcription elongation factors b (P-TEFb), including CyclinT1 and CDK9. These proteins form a complex and phosphorylate the C-terminal domain (CTD) of the

largest subunit of RNA polymerase II, stimulating its elongation activity and allowing the production of full-length HIV-1 transcripts (Fig. 1.5A) [24,25].

Additionally, Rev also has an essential role in HIV-1 replication, regulating the RNA export of single-spliced and unspliced transcripts from the nucleus to the cytoplasm. Rev binds to a loop structure named Rev-responsive element (RRE) located at the 3' of RNA transcripts and interacts with nucleoporin-like proteins at the nuclear pores to initiate a Rev-mediated RNA export cycle (Fig. 1.5B). These interactions allow Rev to export the RNA transcripts to the cytosol without being spliced, enabling production of viral components necessary for particle assembly [26].

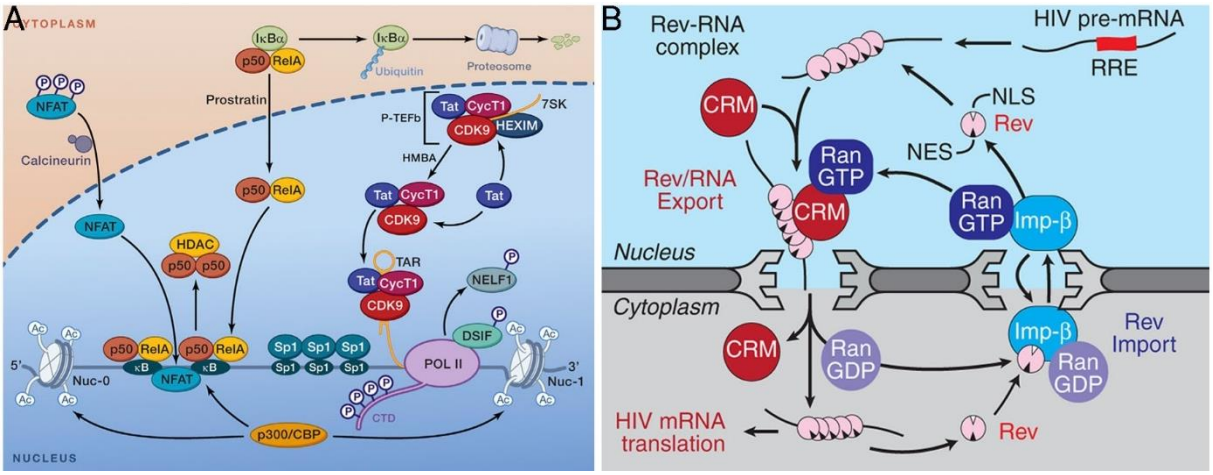


Figure 1.5- Tat and Rev regulatory function on HIV-1 replication. (A) Tat-mediated elongation of HIV-1 transcripts. Following activation of the LTR promoter through binding of NFAT, NF-kB and Sp1 transcription factors, Tat recruits the CycT1/CDK9 complex and other P-TEFb interacting proteins and binds to the nascent RNA chain TAR element. This complex phosphorylates RNA polymerase II CTD, along with negative elongation factors DSIF and NELF, leading to the activation of transcription elongation of HIV-1 transcripts. Source: *Ruelas and Greene 2013* [27]. **(B)** Rev-mediated RNA export cycle. Rev binds to RRE element at viral transcripts and forms a nuclear export complex, by interacting with CRM-1 through the nuclear export sequence (NES) and Ran-GTP. This complex is then exported to the cytoplasm through interactions with nuclear pore proteins. Once in the cytoplasm, Ran-GTP is converted to Ran-GDP, releasing Rev and CRM-1. Rev is imported back to the nucleus by interacting with Importin-β through the nuclear localization sequence (NLS) and Ran-GDP. In the nucleus, Ran-GDP is phosphorylated to Ran-GTP, releasing Rev to begin a new cycle of Rev/RNA export. Source: *Karn and Stoltzfus 2012* [26].

1.1.3. Current treatment to HIV-1 infection

Over the past 30 years, numerous advances in the development of therapies against HIV-1 improved significantly life quality and span of infected patients. Highly active anti-retroviral therapy (HAART) is currently the most successful therapy against HIV-1 infection. HAART consists in a selected combination of three or more antiretroviral drugs that inhibit HIV-1 infection by targeting key steps of the HIV replication cycle (Fig. 1.6). These include nucleoside reverse transcriptase inhibitors (NRTIs) or non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors, entry inhibitors and more recently, integrase inhibitors [28,29]. This antiretroviral cocktail causes a striking decrease in plasma virus levels to below the limit of detection of clinical assays (50 RNA copies/mL) (Figure 8) [30–32].

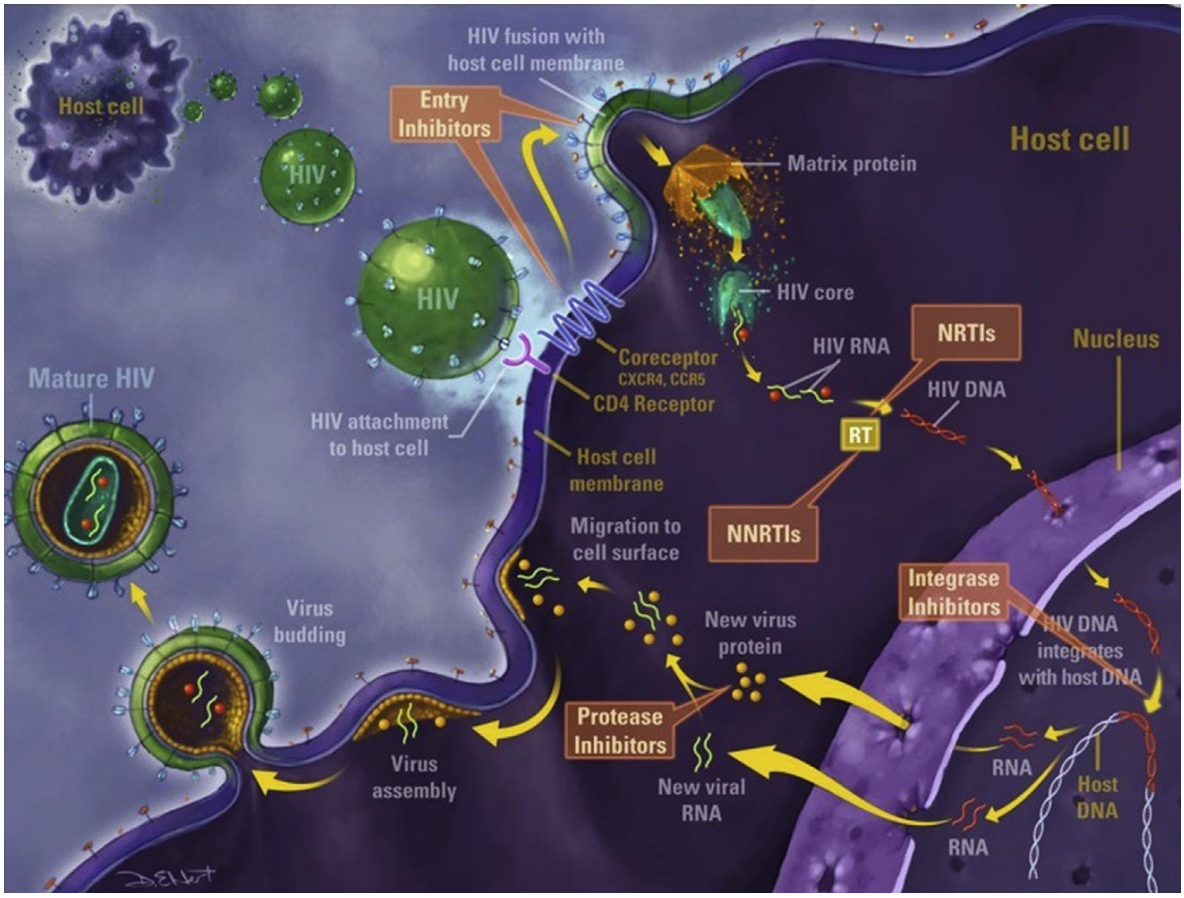


Figure 1.6- HIV-1 replication target points by antiretroviral therapy. Schematic representation of the HIV-1 replication cycle and the key steps targeted by the antiretroviral drugs. NRTIs, Nucleside Reverse Transcriptase Inhibitors. NNRTIs, Non-nucleoseide Reverse Transcriptase Inhibitors. Source: *Kalapila and Marrazzo 2014* [33].

HAART became the standard clinical practice for treatment of HIV-1 infection, allowing a sustained antiviral effect and improving life expectancy of infected patients while considerably reducing the risk of HIV transmission. However, there are adverse effects associated with antiretroviral drugs. Most common issues include hepatic and nephrotoxicity, gastrointestinal symptoms (nausea, vomits, diarrhea), neuropsychiatric problems, myopathy, or decreased bone mineral density. Emergence of resistant virus to some of the antiretroviral drugs is also a potential risk. Patient monitoring for treatment selection is critical to minimize toxicity and optimize adherence [29,33].

In addition, antiretroviral treatment must be maintained throughout the life course while patients have to be constantly monitored for HIV viral load and CD4 cell count from 2-8 weeks to every 3-6 months after HAART initiation [33]. Although no viremia is detected, treatment interruption results in the recovery of viral replication and consequently disease progression (Fig. 1.7). HAART does not cure the patient from HIV-1 infection due to the existence of residual HIV-1 viremia hidden in persistent cellular reservoirs, an infected anatomical site or cell type in which a replication competent form of HIV persists due to low drug penetration or latency [34,35].

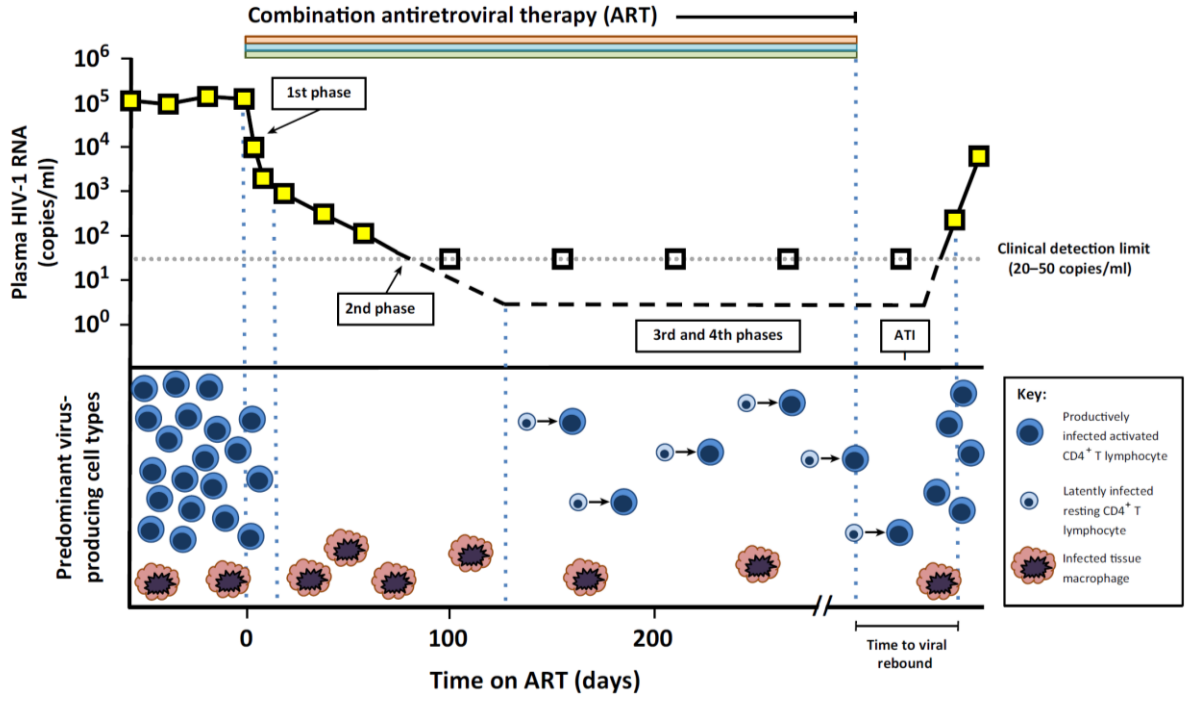


Figure 1.7- HIV-1 kinetics under antiretroviral therapy (ART). The administration of ART leads to a dramatic decay in the plasma HIV-1 viremia. In the first phase, plasma viremia is decreased reflecting the death of the productively infected CD4+ T lymphocytes. The second phase is characterized by persistent replication bellow the detection limit of commercial assays (50 copies/ml) associated with residual infection of long-lived cells, such

as tissue macrophages. Existence of latently infected resting CD4+ T cells which are spontaneously reactivated help maintain the low-level viremia during ART. The contribution of non-T cell reservoirs including chronically infected tissue macrophages to residual viremia remains uncertain. Treatment interruption (in case studies stopping ART with close monitoring) leads to viral rebound and detectable plasma viremia. Source: *Spivak and Planelles 2016* [36].

1.1.4. HIV-1 Latency

One of the primary focus for novel antiretroviral developments, HIV latency is widely considered the main obstacle to achieve a cure for HIV infection. Existence of latent HIV-1 hidden in persistent cellular reservoirs enables them to escape from antiretroviral therapy or host immune response. Even though latent HIV-1 does not replicate in normal conditions, cellular reservoirs can serve as a source of new replicating virus that emerge once its stimulated by specific antigens or cytokines (Fig. 1.7) [27,37]. HIV reservoirs are disseminated into the host and can be found in the blood or bone marrow, and in anatomical sites including the brain, genital tract and gut-associated lymphoid tissues [38].

Latent HIV-1 reservoirs are mainly resting memory CD4+ T-cells established early during acute phase of infection [39]. Residual viremia can be found in other cell lineages such as monocytes-macrophages, astrocytes, follicular dendritic cells and hematopoietic progenitor cells, however their role in the latent HIV-1 reservoir maintenance is not completely understood [34]. The HIV-1 latency can be characterized by two states: pre-integration or post-integration latency (Fig. 1.8). Pre-integration latency is the presence of full-length viral dsDNA that is reverse-transcribed but fails to integrate the host genome, being the most predominant form in untreated patients [40]. The non-permissive environment found in resting memory T cells often blocks HIV-1 replication at this stage, leading to accumulation of linear or circular unintegrated cDNA [41,42]. Stimulatory signals can promote integration of viral cDNA into host genome leading to production of infectious particles, thus this extrachromosomal form of HIV is considered latent [43–45]. However, unintegrated HIV has a very short half-life of 24 hours to few days, limiting the impact of these cells for maintenance of stable reservoirs of HIV [46,47].

Post-integration latency is considered the most relevant form of HIV persistence, established when the viral transcription of a stably integrated provirus is abolished, impairing virus production [40,48]. This is the general case in infected CD4+ lymphoblasts that revert back to a resting memory state and HIV-1 expression is extinguished [27,41]. Their frequency

is rare, in the order of 1 in 10^6 resting CD4+ T cells [49–51]. Resting memory T cells harboring latent integrated proviral DNA are highly persistent in the patient (approximately 44 months), being able to maintain the HIV reservoir for long periods [27]. Once latent cells are stimulated, silenced provirus is reactivated and novel particles emerge to spread infection to new cells [52]. The half-life of HIV latent reservoir is longer than the average life span of the patients, estimated to take approximately 73 years of therapy to eradicate the reservoir [53]. Consequently, HIV-1 latency is considered as the main barrier for complete eradication of the virus from the infected host. Therefore, understanding the mechanisms behind HIV-1 latency is crucial for the development of new drugs or therapies capable of target latent HIV and its cellular reservoirs.

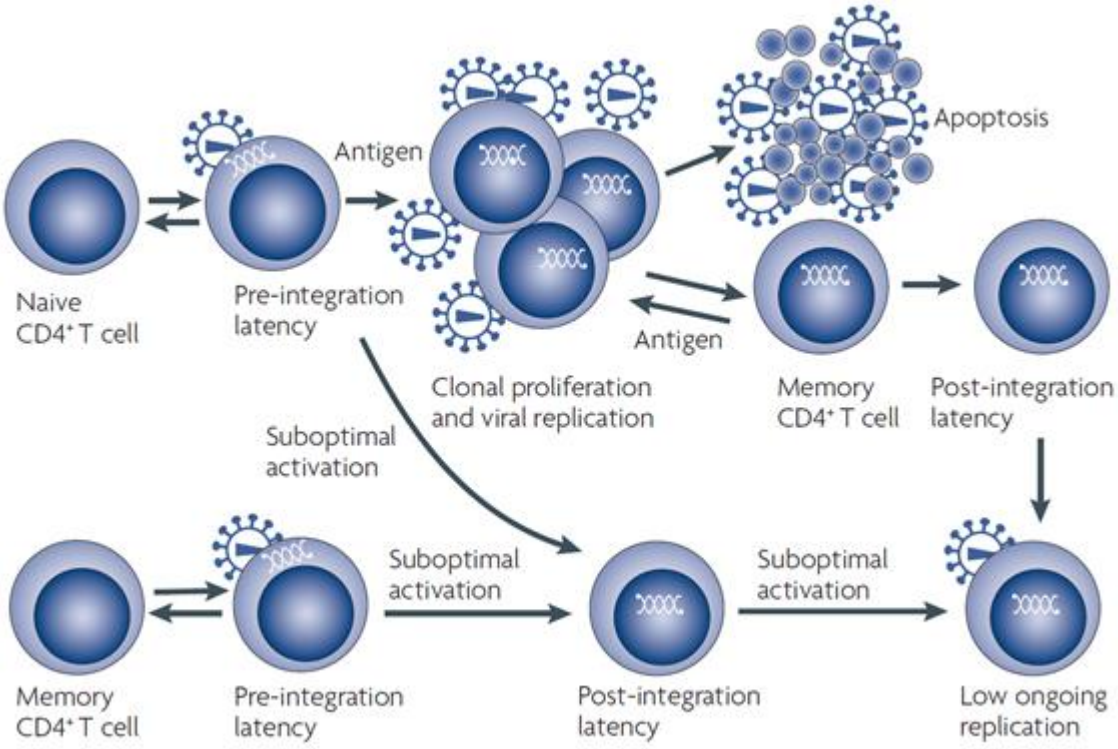


Figure 1.8- Establishment of HIV-1 latency in resting memory CD4+ T cells. Prior to stimulation with specific antigens, naïve CD4+ T cells can be infected and carry unintegrated viral double-stranded HIV provirus which is partially transcribed (Pre-integration latency). Once these cells are stimulated by their cognate antigen, they begin clonal proliferation and promote viral integration into host cell genome and fully activate HIV-1 replication. Over the course of infection, most of these highly HIV-replicating infected cells die through apoptosis. However, some of the effector T cells revert to resting memory T cells, and integrated provirus can be silenced (Post-integration latency). Once the cell is stimulated again with the same antigen, virus replication is reactivated. Alternatively, a resting memory CD4+ T cell can be directly infected and carry unintegrated partially transcribed HIV as it occurs with naïve T cells. Under suboptimal activation, these cells integrate HIV provirus into their genome, inducing a post-integration latent state, which can also promote low-level replication even in the absence of antigen stimulation. Source: *Coiras et al. 2009* [42].

Establishment of HIV-1 latency is mainly determined by the cell transcriptional environment. Several mechanisms involved in the establishment of latency have been identified (Fig. 1.9) [27,37]. Integration in less favorable chromatin environments lead to suppression of HIV-1 transcription by impairing access of key host transcriptional factors to proviral genome [54,55]. Nevertheless, these events occur with low frequency since HIV-1 integrates mostly in actively transcribed regions [56], highlighting the potential role of transcriptional interference on HIV latency. In fact, integration of proviral genome close to highly expressed genes leads to silencing of HIV-1 transcription either through viral promoter occlusion caused by an upstream host gene transcription that displaces critical transcription factors [57,58]; or by collision of RNA Pol II complexes from HIV-1 provirus and a host gene in opposite directions [59–61]. Epigenetic changes caused by DNA methylation [62,63] and histone deacetylation [64] have also a role play in HIV-1 shutdown. Sequestration of host transcription factors in resting CD4+ T cells critical to initiation of HIV-1 transcription, such as NF-kB or NFAT [65,66], or low levels of cyclin T1, part of the P-TEFb complex that assists Tat in the enhancement of HIV transcription elongation [67,68], impairs the completion of the HIV-1 replication cycle. Posttranscriptional mechanisms, including impairment of HIV mRNA nuclear export [69] or innate host miRNA control [70,71] were also identified.

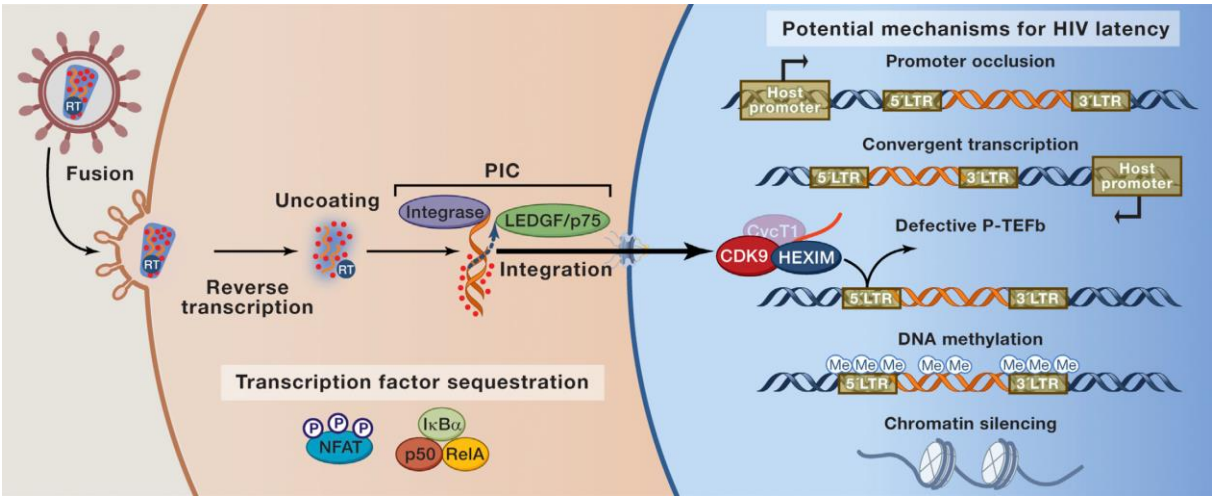


Figure 1.9- Cell transcriptional environment promotes establishment of HIV-1 latency. Schematic representation of the mechanisms associated with HIV latency. Sequestration of NFAT or NF-kB transcription factors essential to HIV transcription initiation leads to silencing of viral gene expression. Transcriptional interference may lead to silencing of viral transcription, either by promoter occlusion when integrated provirus is silenced by an active upstream host promoter and the RNA pol II reads through the HIV-1 LTR; or by convergent transcription, when opposite transcription from host and viral promoter leads to collision of initiating polymerases, inhibiting expression of one or both genes. Low levels of cyclin T1, part of the P-TEFb complex that cooperates with Tat for enhancement of HIV transcription also leads to HIV silencing. Epigenetic mechanisms such as DNA

methylation or histone deacetylation also lead to shutdown of HIV transcription. Integration of HIV provirus into condensed chromatin regions could block access of key transcriptional factors to LTR promoter required to initiate viral transcription. Source: *Ruelas and Greene 2013* [27].

1.1.4.1. Pharmacological approaches to treat latent HIV-1 reservoirs

Because the presence of latent HIV-1 represents an enormous barrier toward its eradication, numerous strategies have been developed to purge it from its cellular reservoirs. In theory, mechanisms associated with HIV latency could be manipulated pharmacologically to reactivate latent HIV-1 and expose these reservoirs to the immune response or cytopathic effects that would clear the infected cells. This strategy is commonly known as "shock and kill" (Fig. 1.10).

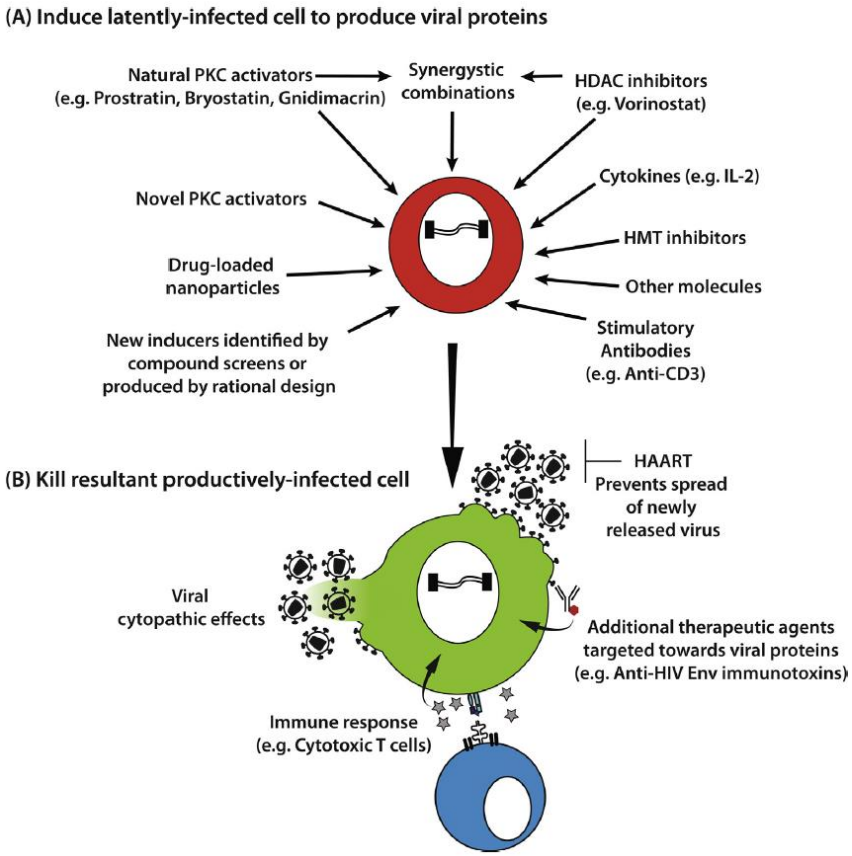


Figure 1.10- "Shock and kill" approach to target HIV-1 reservoirs. HIV latent cells harbor an integrated silent provirus, persisting through antiretroviral treatment or host immune response. However, as simulating memory T cell stimulation, latent reservoirs can be reactivated using a broad panel of stimulating drugs causing cells to actively produce virus. Combinations of these drugs (such as PKC activators and HDAC inhibitors) can synergistically enhance latency reactivation. The production of viral particles can ultimately lead to cell death from the infection or alternatively through immune effector mechanisms or novel approaches targeted to infected cells (such as antibody-drug conjugations). The continued present of HAART will prevent HIV spread to new cells. HDAC, Histone deacetylase. HMT, Histone methyltransferase. PKC, protein kinase C. IL, interleukin. HAART, highly active antiretroviral treatment. Source: *Marsden and Zack 2013* [72].

Considering the mechanisms responsible for the development of latency, methods to force HIV reservoirs to produce viral particles tested so far were mostly based in pharmacological approaches to stimulate T-cells through receptor engagement or downstream activation of signaling pathways [52]; or epigenetic remodeling through inhibition of histone-modifying [73] or DNA methylation [62,63] enzymes.

Strategies that simulate the natural activation of T-cells through antigen and cytokine stimulation should reactivate latent expression similarly as occurs in the patient. Treatment with anti-CD3 chain of T-cell receptor and IL-2 triggers cell activation along with expression of latent HIV [74], nonetheless it reveals toxic effects resulting in long-lasting depletion of CD4+ T cells in peripheral blood and lymph nodes [75]. IL-7 cytokine causes a milder activation of T-cell but sufficient to trigger HIV expression [76,77] yet homeostatic proliferation caused by this cytokine proved responsible for maintenance of HIV reservoir [78]. Protein kinase C (PKC) signaling pathway is connected to activation of transcription factors necessary to induce HIV transcription. PKC agonists such as prostatin [79,80] or bryostatin [81] reactivate HIV transcription in latent T cells through induction of NF-kB. Although these agents cause a striking increase in HIV transcription, they also trigger undesired global activation of immune response with a toxic inflammatory response [42,52].

Histone deacetylases (HDACs) promote hypoacetylation of nucleosomes with compaction of chromatin, contributing to HIV reservoirs by restricting access to transcription factors [37]. HDAC inhibitors, such as valproic acid [82] and vorinostat [83,84] are promising agents capable of inducing viral gene expression without global T cell activation by disrupting recruitment of HDAC proteins to the HIV long terminal repeat (LTR) promoter. Both HDAC inhibitors are able to increase HIV transcription from resting T cells of aviremic infected patients, however no significant reduction of the reservoir size was observed [85–88]. DNA methylation of CpG dinucleotides at HIV 5'LTR is another relevant epigenetic mark responsible for HIV silencing by impairing binding of critical transcription factors like NF-kB [89]. Treatment with 5-aza-2'deoxycytidine (aza-CdR), an inhibitor of DNA methyltransferases, increases viral gene expression of latent cells [62,63]. Nevertheless, this treatment is limited by the low level of 5'LTR methylated events that actually contribute to the HIV reservoir [90]. In conclusion, despite the evidence that these drugs can induce latent viral expression, none of the strategies mentioned above could eradicate latent HIV from cellular reservoirs, and some of them were associated with undesirable and toxic generalized immune activation.

In addition to these limitations, the immune system of infected patients lacks a robust cytotoxic T lymphocyte (CTL) response essential to clear the reactivated cells, suggesting that reactivating HIV reservoirs alone is not sufficient to promote their elimination. Stimulation of CTLs with HIV-specific antigens prior to reactivation of latent infected cells seem critical for efficient eradication of these reservoirs [91]. Another major concern is the high frequency of CTL escape mutations detected in resting CD4+ T cells from patients receiving antiretroviral treatment during the chronic phase of infection in a study by Deng *et al.* 2015 [92]. Emergence of HIV reservoirs harboring escape mutations seem to be driven by the selection pressure caused by CTL response during the acute phase of infection leading to selected growth of CTL escape variants, posing a major challenge to the reservoir eradication [92,93]. These limitations highlight the need for the development of alternative and more targeted strategies against latent HIV-1 and its cellular reservoirs.

1.2. Gene Therapy

Emerging in the late 1980s, gene therapy has promised to innovate the field of molecular medicine, providing novel ways to correct a wide range of genetic or acquired diseases. Gene therapy can be described as the transfer of nucleic acids (DNA or RNA) to treat, cure or prevent human disorders. Most gene therapeutic applications aim to correct a cell harboring a defective gene responsible for the syndrome by introducing a functional copy of the gene, or reduce levels of a harmful gene product by targeting its transcription using sophisticated tools [94].

Initially focused on primary immune-deficiencies, for which there was lack of alternative treatments, gene therapy quickly spread out to target other disorders related to cancer, heart failure, neurodegenerative or metabolic disorders, so far with approval of over 2300 clinical trials for gene therapeutic applications (Fig. 1.11; The Journal of Gene Medicine Clinical Trial site).

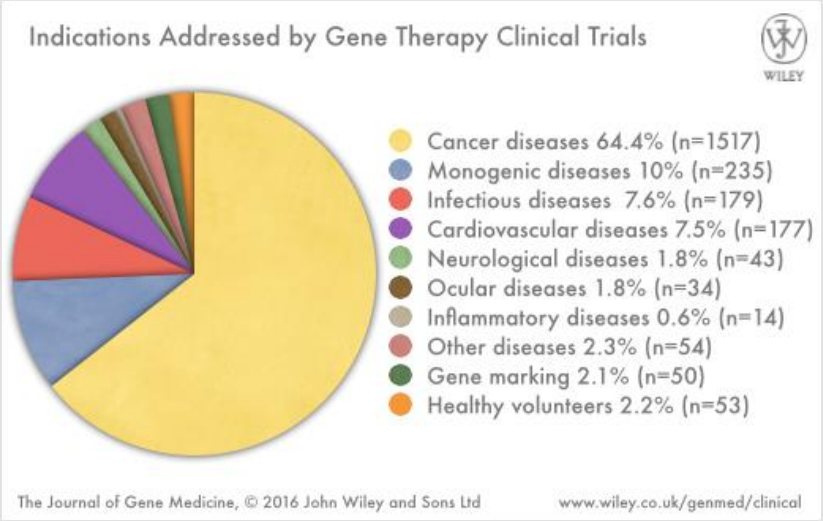


Figure 1.11- Indications addressed by gene therapy clinical trials. Global representation of worldwide gene therapy clinical trials distributed by targeted diseases. Source: The Journal of Gene Medicine Clinical Trial site (www.wiley.co.uk/genmed/clinical).

Gene therapeutic applications can be implemented *in vivo*, by direct introduction of the therapeutic gene into the patient, or *ex vivo*, by correcting the defective cells outside the patient (Fig. 1.12).

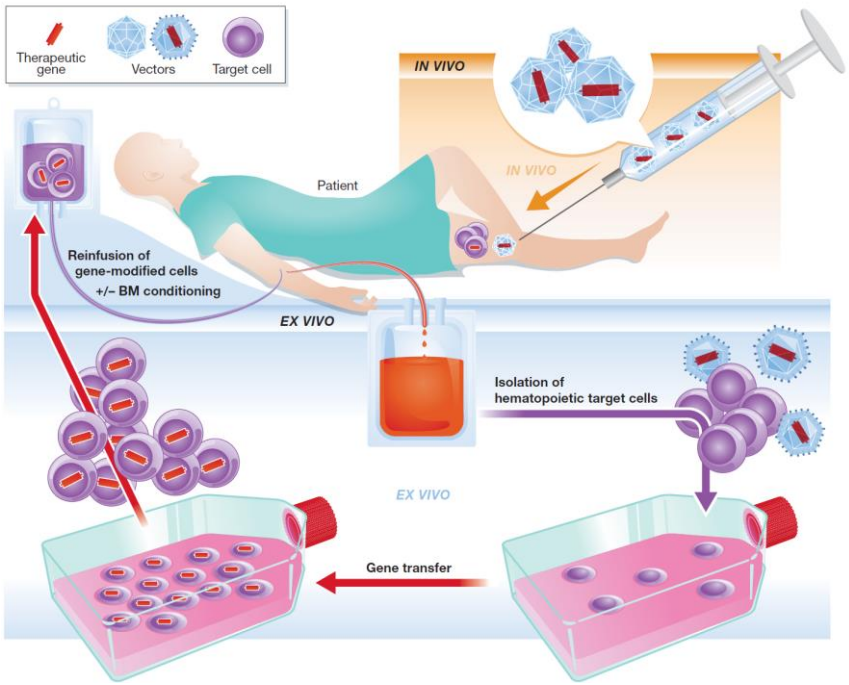


Figure 1.12- Gene therapy *in vivo* vs. *ex vivo*. For *in vivo* applications, correction of the patient defective cells is promoted by direct introduction of the therapeutic gene into the body (e.g. muscle, liver) through a delivery vector (viral or non-viral). For *ex vivo* applications, cells (e.g. hematopoietic stem cells from the bone marrow) are collected from the patient, genetically modified outside the body and reintroduced into the patient as an autologous transplant. BM, bone marrow. Source: *Kaufmann et al. 2013* [94].

Gene transfer to target cells is mediated through vectors that can surpass the complex cell and tissue barriers and deliver the new genetic material into the target cell without disrupting essential regulatory mechanisms (Fig. 1.13). These vectors are classified as viral or non-viral. Ideally, vectors for gene delivery should possess some of these critical attributes: ease production on a commercial scale with low costs associated; sustain long-term gene expression; present low immunogenicity; programmable to be retargeted to certain cell types or tissues; package large genetic material; replicate or segregate during cell division for long-term effects; and transfect either dividing or non-diving cells [95]. Although non-viral methods (e.g. naked DNA, liposomes or cationic polymers) present some advantages such as lower cost and immunogenicity as well as higher packaging capacity [96], viral vectors continue to be the most exploited vehicles, dominating gene therapy clinical trials due to their superior efficiency in gene delivery [95,97].

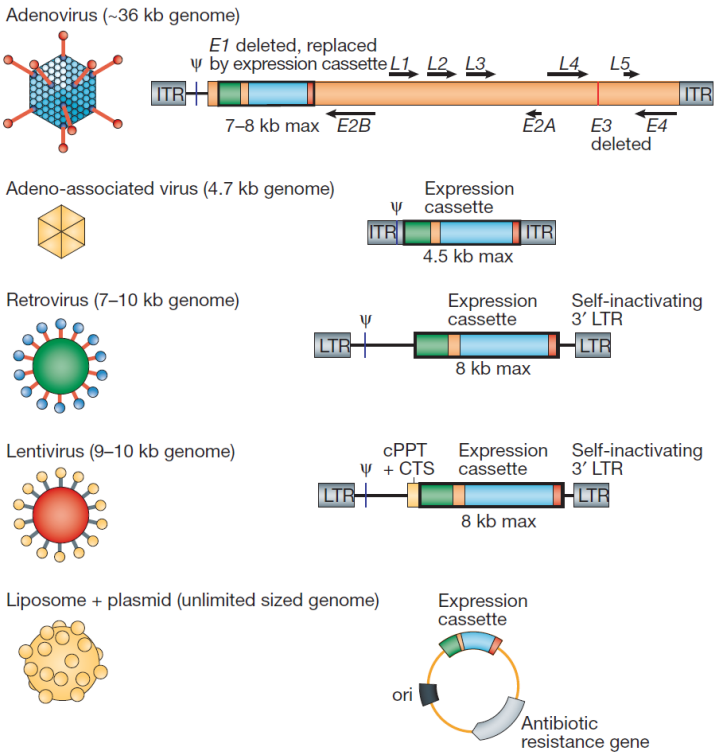


Figure 1.13- Gene-transfer vectors typically used for therapeutic applications. Therapeutic gene is incorporated into an expression cassette, flanked at 5' by a promoter and at 3' by a polyadenylation site. This expression cassette is packaged into a viral or non-viral vector. Viral vectors implemented are mostly composed by adenovirus, adeno-associated virus (AAV), retrovirus or lentivirus. Non-viral vectors deliver plasmid DNA or mRNA either naked or incorporated into liposome or cationic based particles. The limit of expression cassette size is indicated for each vector. ITR, inverted-terminal repeats. Ψ, packaging signal. E1-E4, early genes. L1-L5, late genes. LTR, long-terminal repeats. cPPT, central polypurine tract. CTS, central termination sequence. Ori, bacterial origin or replicarion signal. Source: *Sheridan 2011* [98].

Viral vectors are derived from natural viruses by splitting their genetic components and replacing the pathogenic elements for a therapeutic gene. These components are introduced *in trans* to a packaging cell line to produce viral particles that will deliver the therapeutic gene to the target cell but lacks the components necessary for further propagation to other cells [95]. Viral vectors derived from adenovirus, adeno-associated virus, retrovirus or lentivirus family (Figure 14) are so far, the most relevant vehicles implemented for gene therapy applications.

Replication-defective adenoviral vectors (AdVs) are derived from the *Adenoviridae* family of double-stranded DNA non-enveloped viruses able to transduce both dividing or non-dividing cells, remaining in the nucleus as non-integrated episome [95]. More than 50 types of adenovirus have been identified that interact with a broad range of cell receptors and are associated with asymptomatic infections specially in the respiratory tract, intestine, eyes or liver, possibly lethal in immunocompromised individuals [99]. AdVs can be produced at high titers and their large dimension enables packaging of large or multiple transgenes – up to 37 Kb if most viral components are removed from the vector - into viral particles that sustain high-level expression in target cells without risk of insertional mutagenesis [100]. These key abilities made these viruses the most used vector for gene therapeutic applications, with over 500 clinical trials involving adenovirus delivery. In addition to gene delivery, AdVs are also exploited as prophylactic vaccines or oncolytic viruses. Human trials using AdVs were carried for treatment of cardiovascular disorders, cystic fibrosis, hemophilia A or viral infections [101]. However, most AdV applications focus on cancer treatment, opening up the door for the first world-wide gene therapy approved in China for treatment of head and neck carcinoma (Gendicine) [99]. The main hurdle with AdVs is the *in vivo* cellular and humoral immunological response to viral particle components or transgenes which can trigger fatal adverse effects [102], but also cause loss of long-term transgene expression [103,104]. This adverse effect limits adenoviral gene transfer applicability *in vivo* particularly for cases that only require sustained gene transfer or multiple doses of vector injection.

Similar to adenovirus, adeno-associated virus (AAV) efficiently transduce both dividing and non-dividing cells but present far less immunogenicity, being widely explored for *in vivo* applications [105]. These single-stranded DNA particles from the *Parvoviridae* family are small and non-enveloped, persisting in the target cell mostly in the episomal form, able to replicate only in the presence of helper virus (adenovirus or herpes simplex virus) [106]. Viral tropism is determined by the capsid composition that mediate binding to cell surface-receptors, promoting endocytic uptake [107]. The discovery and engineering of several naturally

occurring AAV serotypes with distinct viral tropism greatly expanded available options for targeting a broad range of tissues, including the brain, liver, eye or muscle [108]. Recombinant AAV (rAAV) vectors have shown tremendous clinical efficacy for treatment of numerous genetic diseases such as haemophilia B, Leber's congenital amaurosis type II, chloroideremia and lipoprotein lipase deficiency [109], the latter culminating in the first gene therapy product approved in the European Union by 2012 (Glybera) [110]. Challenges faced with AAV come from their reduced size which limits the packaging of large transgenes [111]. Additionally, although it can integrate genome at low frequencies through NHEJ-repair [112], AAV do not possess an integration machinery, therefore unable to sustain gene transfer in proliferating populations relevant for gene therapeutic applications, such as malignant or hematopoietic cells.

Retroviral vectors (RVs) were among the first to be implemented in gene therapy clinical trials, particularly due to its unique machinery that promote stable gene transfer into dividing populations. Retrovirus are RNA-virus that retrotranscribe its genome and integrate it into the target cell chromosome, therefore propagating long-term expression to its progeny [95]. Gene therapy through murine leukemia-derived gamma-retroviral vectors (γ-RVs) took significant importance for translation of *ex vivo* cell therapies, in particular for hematopoietic stem cell (HSC) transplantation. CD34+ HSCs can be collected from the patient and genetically modified with a transgene to correct the defective cell, emerging as an alternative therapeutic option for treatment of several blood and immune, or lysosomal storage related disorders [113]. Once reinfused in the patient, these genetically modified pluripotent cells allow long term repopulation and differentiation, which could represent a long-life treatment for the patient. Opposed to using HSC from healthy donors, reinfusing autologous (self) HSC from the patient has the advantage of preventing immune incompatibilities which could lead to rejection of engraftment or graft-versus-host disease. γ-RVs were naturally adopted for HSC therapy due to the requirement for transgene integration and long-term expression that is propagated to the reconstituted hematopoietic lineage [113]. Early clinical trials used HSCs genetically modified with γ-RVs to treat two types of severe combined immunodeficiency (SCID), X-linked (SCID-X1) [114] and adenosine deaminase-deficiency (ADA-SCID) [115] with outstanding results, clearly showing the potential benefit of autologous HSC gene therapy to correct these inherited genetic diseases. In 2016, the first *ex vivo* HSC gene therapy was approved by the European Union for treatment of ADA-SCID (Strimvelis), after 18 infants showed clear marks or long term benefits and survival from this treatment [116].

Despite their tremendous success in early gene therapy clinical trials, cases of children developed leukemia were reported from this vector transfer [117]. The abnormal condition derived from retrovirus-mediated insertional mutagenesis that caused activation of a particular oncogene [118,119]. In addition, retrovirus are unable to transduce non-dividing cells, which difficult gene transfer into primitive progenitor cells [113]. These hurdles clearly marked the need for continuous development of more efficient but also importantly safer vectors for gene transfer.

1.2.1. Lentiviral gene therapy

Early gene therapeutic applications derived in a large portion from murine leukemia-derived γ-RVs gene delivery which were particularly used for their ability to integrate the cell genome, allowing life-long treatment for the patient [113]. However, reports of several cases of hematological malignancies were associated with insertional activation of oncogenes by these vectors [117,118,120]. In addition, this class of retroviruses is limited by its inability to infect non-dividing cells, impairing their application in a wide variety of target cells and tissues [121–123].

HIV-derived lentiviral vectors (LVs) overcame these limitations as these viruses do not require cell division to transduce and integrate the target cell genome [124], resulting in more efficient gene transfer and robust gene expression as compared to γ-RVs. LVs have also been associated with less risk of insertional genotoxicity and potential to trigger oncogenesis due to differences in integration profile [125–128]. Introduction of self-inactivating (SIN) LTR that removes transcriptional elements from both LTRs during retro-transcription further enhanced safety of lentivirus transduction [127]. Furthermore, LVs present reduced immunogenicity upon *in vivo* injection [129,130] compared to other frequently used virus family for gene therapy such as adenovirus [131] or adeno-associated virus (AAV) [132]. Substitution of the virus natural envelope for vesicular stomatitis virus G protein (VSV-G) releases lentivirus of their dependence of CD4 receptor to target a wide range of host cells [124]. VSV-G pseudotyped lentivirus bind LDL receptors ubiquitous expressed across most cell types [133] and also confer high stability to viral particles that enable its concentration to higher titers for efficient gene transfer [134], commonly used as the standard envelope used for *ex vivo* lentiviral gene therapy.

Preclinical research has improved vector safety and reduced possibility of generation of replication-competent by removing viral genes and regions important for HIV pathogenesis but unnecessary for gene transduction [135,136]. These modifications to the lentivirus genome led to the development of second-generation and third-generation of lentiviral systems which are now broadly used to deliver transgenes (Fig. 1.14).

Lentivirus has tremendous potential in clinical applications, nevertheless potential side effects derived from insertional mutagenesis might still occur with these vectors [61,137]. In certain cases, correction the defective cell only requires transient transgene expression. To avoid genotoxicity, LVs can be modified to abrogate integration of the provirus into the cell chromosome, generating integration-deficient lentiviral vectors (IDLV). Gene expression from IDLVs is driven from unintegrated retroviral episomes that can remain in the nucleus for long periods as a linear or circular form [138]. IDLVs are generated by incorporating class I mutations in the *pol* region of the packaging construct that disable viral Integrase activity (Fig. 1.14).

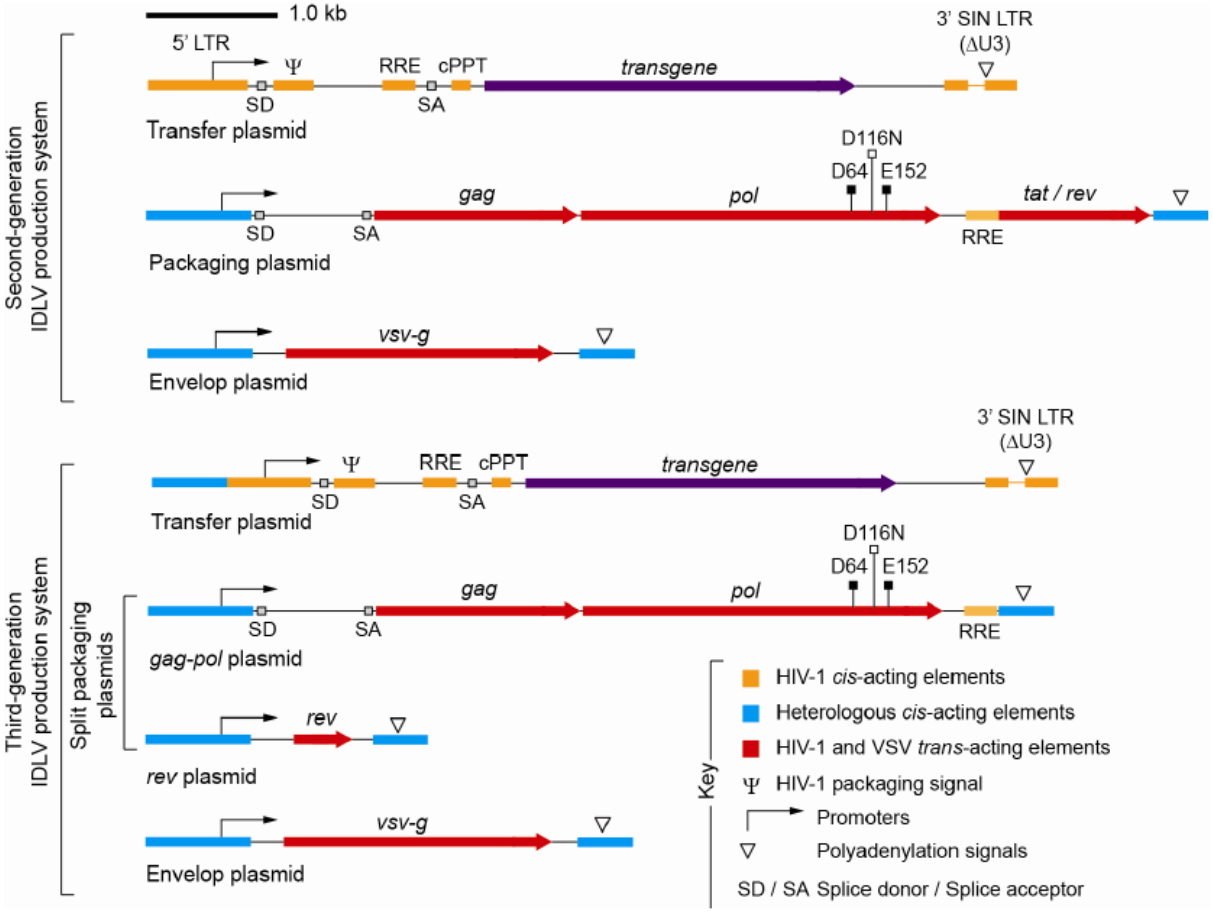


Figure 1.14- HIV-1 based lentivirus production system. Schematic representation of the second and third-generation lentiviral production system. HIV-derived lentiviruses are assembled by co-transfecting producer cells, generally HEK293T cells, with transfer, packaging (*gag-pol*) and envelope constructs. Transfer construct harbors the therapeutic transgene flanked by 5' and 3' long terminal repeats (LTR). Gene expression can be regulated by the 5'LTR or a heterologous promoter incorporated upstream of transgene. Third-generation transfer plasmid contain a hybrid 5'LTR composed of partial HIV sequence necessary for integration preceded by heterologous transcriptional elements (generally from cytomegalovirus) that confer Tat independence to vector genome expression. Self-inactivating (SIN) vectors have their LTR enhancer region deleted (ΔU3) to reduce potential recombination events that generate replication-competent virus. Transfer construct also contains packaging signal (Ψ) for genome encapsidation, the Rev-responsive element (RRE) that mediates nuclear export of unspliced and single-spliced transcripts, and the central polypurine tract (cPPT) for transduction enhancement. To increase biosafety of lentivirus production, packaging construct of third-generation system is disposed of *tat* and *rev* coding sequences, driving expression of particles components Gag and Pol alone. In the third-generation system, *tat* is not necessary to drive expression of the transfer plasmid, while *rev* encoding plasmid is delivered *in trans* to drive efficient translation of RRE-containing transfer and packaging constructs. The envelope plasmid generally encodes the vesicular stomatitis G protein (VSV-G) that confer pantropic host range to viral particles. Integration-deficient lentiviral vectors (IDLV) can be produced by delivering packaging constructs that incorporate class I amino acid substitutions (D64, D116 and E152) of the Pol polyprotein that are located at the catalytic core of viral Integrase and disable its function. Source: *Chen and Gonçalves 2016* [139].

Remarkable efficiency and safety of engineered lentivirus led to the implementation of these vectors for clinical applications in human disorders [140]. The first clinical trial in humans involving lentiviral delivery used genetically modified autologous CD4+ T cells (VRX496-T) transduced with an LV encoding an antisense RNA targeting the HIV envelope. Multiple engraftment of vector-modified T cells in HIV aviremic patients reduced viral load in 6 out of 8 subjects without any signs of clonal transformation or integration near oncogenes [141], demonstrating the potential of lentivirus for gene therapy. The stable expression and safety profile of lentiviral transduction in T cells played a key role in the progress of cancer immunotherapy by engineering T lymphocytes to express a transgenic T-cell receptor (TCR) or chimeric antigen receptor (CAR) that target and eliminate malignant cells [142].

More importantly, LV mediated gene transfer dramatically improved efficiency of HSC therapy due to their superior and safe gene delivery in more primitive progenitor cells as compared to early generation γ-RVs [140]. Lentivirus succeed in obtaining robust levels of gene transfer in HSCs, having so far achieved tremendous efficacy in clinical trials that treated patients from fatal disorders such as X-linked adrenoleukodystrophy [143], metachromatic leukodystrophy [144], or Wiskott-Aldrich syndrome [145]. Other ongoing trials for X-linked severe combined immunodeficiency (SCID-X1) [146] or hemoglobinopathies [147] also promise to follow the same level of success, raising expectations about a wave of market approval of novel gene therapies through lentiviral *ex vivo* correction of HSC.

In addition to correct stem cells for gene therapeutics, lentivirus have also been exploited to generate *in vitro* this scarce and hardly accessible source. Lentiviral delivery of reprogramming factors, also known as “Yamanaka factors” [148], can reprogram somatic cells back into pluripotent state, generating induced pluripotent stem cells (iPSC) [149]. This method promises to revolutionize regenerative medicine by producing healthy stem cells from a nearly unlimited source that could in principle be programmed to correct and generate any cell type or tissue [150]. However, reprogramming factors must be expressed during a short window period (20-30 days) just enough to induce pluripotency but maintain their ability to differentiate. This requirement led to development of a next-generation inducible lentivirus for specific control over expression of reprogramming factors, incorporating Cre-recombinase excisable cassettes with a doxycycline-inducible promoter for transient transgene expression [151]. Nevertheless, concerns about the unpredictable effect of genomic scars remaining after lentiviral integration appeal to investigate non-integrative vectors as an alternative and safer method to reprogram iPSC [152].

It was mentioned that lentivirus can be engineered to avoid genotoxicity associated with integration into cell chromosome, while maintaining episomal transgene expression. IDLVs provide transient expression in proliferating cells at lower levels compared to its integrative counterpart. Nevertheless, these non-integrative vectors have shown tremendous persistent expression both *in vitro* and *in vivo* – up to several months in some cases – after transduction of non-dividing tissues, including the eye, brain, spinal cord, muscle and to a less extent the liver [138].

Transient gene transfer by IDLV have proven useful for several clinical applications, particularly in cases for which sustained expression is detrimental to the target cells. Overexpression of a truncated form of CXCR4 receptor by IDLV transduction promotes mobilization of peripheral blood stem cells after interaction with its correspondent SDF1 ligand, enhancing HSC engraftment in mouse models of SCID [153]. Vaccination through IDLV mediated intracellular expression is able to stimulate antigen-specific cellular or humoral immune responses against HIV [154] or hepatitis B [155]. Efficient knockdown of target genes by IDLV delivery of short hairpin RNAs demonstrate the potential of this technology for modulating gene expression through RNA interference [138]. One major application of IDLV transduction is their potential to correct defective genes *in situ* through genome engineering methods, acting not only for transient expression of targeted endonucleases but also encoding healthy gene templates for homologous recombination [156] (further discussed in chapter 1.2.3.3.). In the future, methods that promote IDLV episomal replication to sustain gene expression in proliferating populations, such as inclusion of viral SV40 origin of replication [157] or eukaryotic scaffold/matrix attachment regions (S/MAR) [158], will surely broaden the range of IDLV gene therapeutic applications while maintaining the non-integrative vector biosafety.

Most successful lentiviral applications focus on *ex vivo* correction of defective cells due to their limitation to target specific cell types *in vivo.* Efficient gene delivery to the target cell population is of major importance to avoid wasting vector particles to irrelevant cells, potentially triggering side effects if delivering a toxic gene or possibly targeting harmful cells such as antigen-presenting cells. For this purpose, strategies have been developed to generate receptor-targeted lentivirus by engineering viral surface to adapt targeting ligands that mediate transduction to specific cell population. Proof-of-principle of targeted LV transduction was demonstrated in tumor or endothelial tissue, nervous or hematopoietic system, as well as distinct lymphocyte cell subtypes [159]. This method holds great potential for the direct

application of lentivirus *in vivo* for treatment of relevant human diseases, including HIV infection.

1.2.2. Gene-therapy against HIV-1

Over recent years, gene-based therapies have gained increased relevance as a promising alternative to treat HIV-1 infection. Although antiretroviral drugs have significantly improved quality of life of treated patients, these have failed to eradicate HIV infection. Gene therapy offers alternative methods to eliminate HIV infection in a targeted and more efficient manner by using a wide panel of sophisticated tools that can target key aspects of HIV beyond the scope of antiretroviral drugs.

The concept of gene therapy against HIV-1 consists in delivering therapeutic genes to inhibit HIV replication or promote elimination of HIV infected cells. Strategies developed so far aimed to turn cells resistant HIV-1 replication, promote apoptosis of HIV-1 infected cells or immunize against HIV-1 antigens to engineer anti-HIV cytotoxic cells (Fig. 1.15) [72,160,161]. Most of these gene therapeutic applications are divided into protein-based therapies (e.g. transdominant negative mutants, toxins, and targeted endonucleases) and RNA-based strategies (e.g. antisense, ribozyme, RNA aptamers, RNA decoys, short-hairpin RNA and small-interfering RNA) [72,160,162].

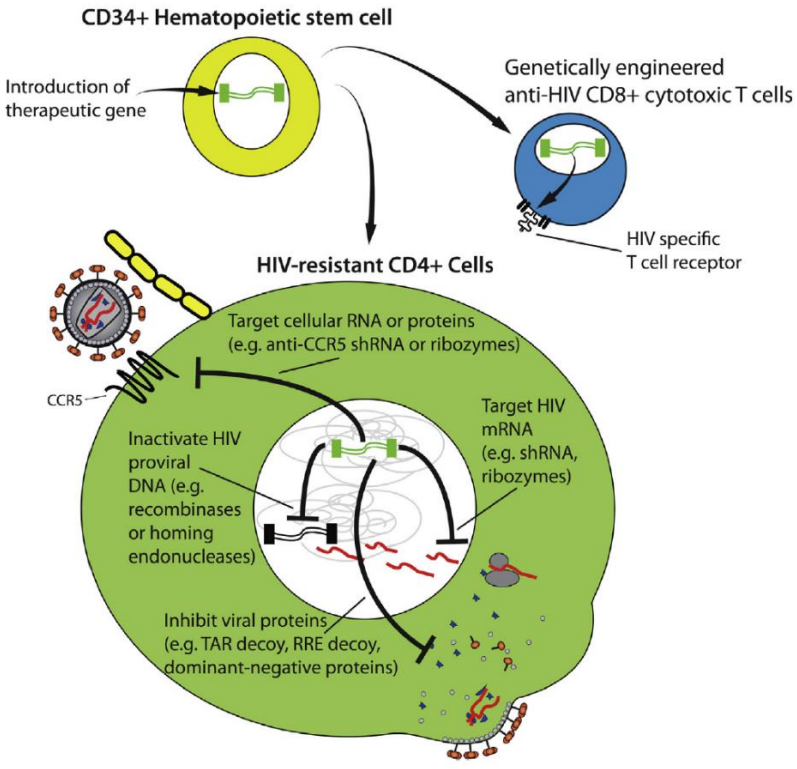


Figure 1.15- Gene therapy approaches targeting HIV-1 infection. Schematic representation of relevant HIV-1 gene therapeutic applications developed against HIV-1 infections. Source: *Marsden and Zack 2013* [72].

1.2.2.1. Engineering HIV immunity

During chronic infection, the host immune response mediated by CD8+ cytotoxic T lymphocytes (CTL) is not able to clear the HIV infected cells. Supplementary stimulation of anti-HIV immune response could enforce more efficient responses when battling HIV infection [91]. Initial efforts aimed to enhance antiviral humoral immune response through passive administration of HIV neutralizing monoclonal antibodies [163,164], or enhance the existing cellular immunity by collecting and expanding anti-HIV CTL *ex vivo* followed by reinfusion into the patient [165–167]. Although these approaches were proven safe and successfully stimulated host immunity against HIV infection, they failed to significantly reduce the patient viral load [168]. Immune-based gene therapy has provided novel methods for this goal, by genetically manipulating the natural immune system to enhance the anti-HIV cellular immune response [168]. Gene therapy approaches to engineer HIV immunity consist in redirecting the peripheral blood CTLs cells to target HIV more effectively. Peripheral CTLs cells have been genetically modified to express a transgenic full T-cell receptor or a modified chimeric receptor at the surface to target them to HIV-antigen expressing cells [169].

Incorporation of transgenic T-cell receptor (TCR) selective for HIV-antigens presented by human leukocyte antigen (HLA) has proven to enhance redirection of peripheral T cells to suppress HIV infection [170]. In this approach, HLA-restricted peptide-specific TCR are collected from reactive T cells of infected individuals that show robust CTL-mediated immune response against HIV. These TCR are then molecularly cloned, modified and selected for higher affinity towards HIV peptide-HLA [170–173] and transferred by retroviral or lentiviral gene delivery into patient's peripheral T cells *ex vivo* to enhance the anti-HIV immune response [168].

Opposed to transgenic TCR engineering, chimeric antigen receptor (CAR) uses an exogenous antigen-binding domain fused to the zeta chain signaling domain of the T-cell receptor that triggers cell immune activation once the antigen is recognized. CAR-T cells have gained awareness in the treatment of hematological malignancies, achieving outstanding results in remission of B-cell acute lymphoblastic leukemia for several patients, consequently considered one of the breakthroughs of 21st century [174]. The use of chimeric receptors should enable a broader recognition of HIV-antigen expressing cells by avoiding the obstacles associated with human leukocyte antigen (HLA) restriction observed with full-length TCR [168]. Early studies using CARs based on CD4 epitope directed to gp120 have presented

increases in the CD4 T cells and moderate decreases of viral reservoirs in HIV infected patients infused with these *ex vivo* modified CAR-T cells [175]. Following the development of broadly neutralizing antibodies and design of new-generation of CARs, recent data have demonstrated improved efficacy towards elimination of HIV-Env expressing cells and demonstrating the potential of CAR T cells for treatment of chronic HIV infection [176–179].

Limitations with these approaches arise mainly from the *ex vivo* manipulation of these cells which could interfere with their normal function or life span once reinfused in the patient [180]. The presence of natural TCR in these modified cells could also generate auto-immunity by self-reactive T cells as a result from cross-pairing between endogenous and the transgenic TCRs [181,182].

1.2.2.2. HIV suicide gene therapy

Besides engineering peripheral T cells to selectively eliminate HIV infected cells, gene therapy can also provide tools to cause the infected cell to kill itself, an approach known as "suicidal gene therapy". This approach is frequently used to target cancer cells [183] and consists in delivering toxins or suicidal genes that induce cell apoptosis once its expressed. To target HIV infected cells and avoid harming heathy uninfected cells, expression of these suicides genes is conditioned to elements responsive to virus infection.

Selective killing of HIV-1 infected cells is generally achieved by delivering lentiviral vectors encoding suicidal genes responsive to Tat/Rev proteins. These lentiviral vectors were initially developed as sensors for HIV infection by encoding a gene reporter which expression was conditioned to the Tat-mediated enhancement of gene expression from the LTR promoter [184–187]. However, leaking of expression from Tat-independent basal transcription lead to development of new-generation of reporter vectors that also conditioned gene expression to the Rev-mediated exportation of unspliced transcripts for translation in the cytoplasm [188] (Figure 1.16).

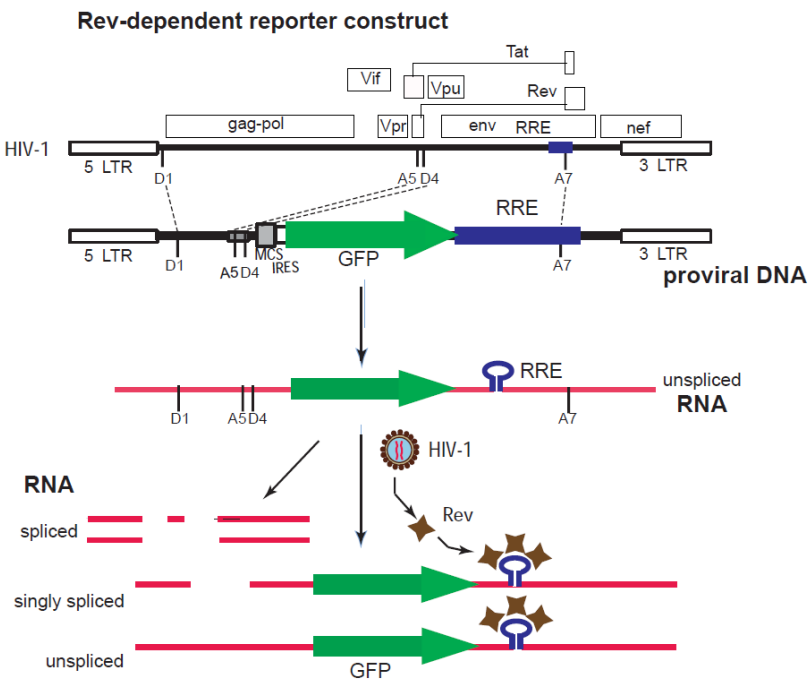


Figure 1.16- Rev-dependent lentivirus reporter for HIV-1 infection. HIV-reporter vector was constructed based on HIV-1 genome elements. Lentiviral construct contains both LTRs, 5' end of *gag* gene, virus splice donors (D1, D4) and acceptor (A5, A7) sites, and a segment of *env* gene containing the Rev-response element (RRE). Green fluorescent protein (GFP) is present in this vector as gene reporter for HIV infection. In the absence of HIV infection, transcripts generated by Tat-independent basal transcription are immediately spliced, removing the region encoding the GFP open reading frame. Once the cell is infected by HIV, Rev expression promotes export of single and non-spliced transcripts from the nucleus to the cytosol, thus leading to gene reporter expression. Source: *Wu et al. 2007* [188].

Rev-dependent lentiviral vector appeared to be absent of HIV-independent background activity, and consequently strategies for selective killing of HIV infected cells were developed by introducing suicidal genes in these HIV-reporter constructs [189,190]. Although these reports have proven that it is possible to achieve specific elimination of HIV-infected cells using a Rev-dependent lentiviral vector, cell death is completely dependent of the presence of an active replicating virus. HIV-1 cellular reservoirs are not targeted, due to lack of Tat/Rev expression. Prior reactivation of latent transcription is required to induce viral gene expression, activating the Tat/Rev-dependent circuit that leads to elimination of the infected cell.

1.2.2.3. HIV intracellular immunization

In general, most gene therapeutic approaches that reached clinical trials aimed to turn cells resistant to HIV infection. Development of innovative tools that target key points of HIV replication cycle can be genetically transferred into the patient cells and generate intracellular immunization (Fig. 1.17).

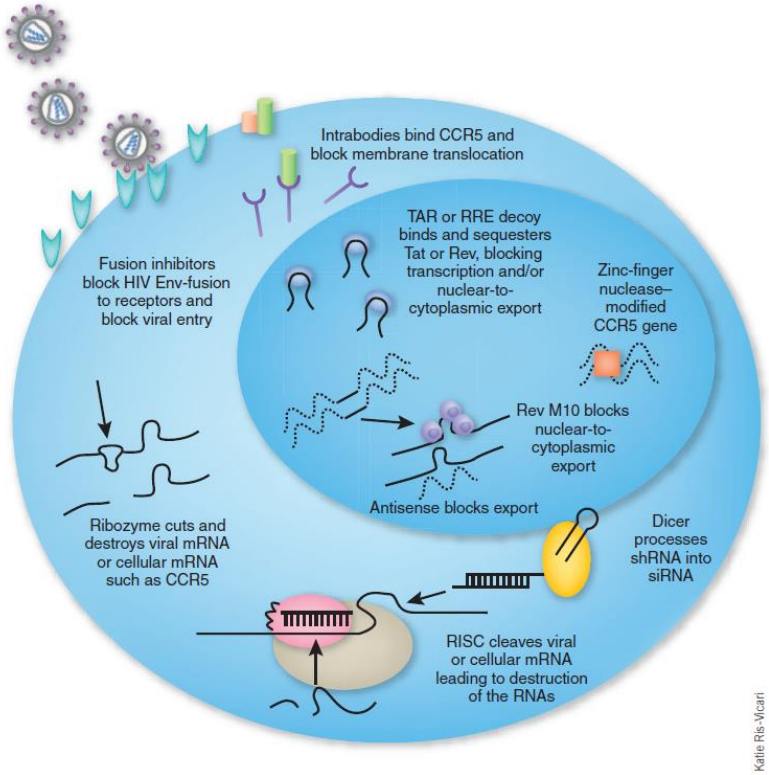


Figure 1.17- Gene therapeutic strategies to engineered HIV resistant cells. Schematic representation of relevant antiviral gene therapy approaches that target viral replication cycle. Source: *Rossi et al. 2007* [191].

HIV proteins Tat and Rev have a critical role in the transition from early to late stage of replication cycle by respectively transactivating viral transcription and mediating exportation of single-spliced and unspliced transcripts (see chapter 1.1.2.1. for more details). Several gene therapy approaches have focused on these regulatory proteins by targeting both protein expression and function. Delivery of syntethic small-interfering RNA (siRNA) targeting both Tat and Rev mRNA to degradation were able to block their protein expression and inhibit viral replication in human T-cell lines and primary lymphocytes [192]. Similar approaches using lentiviral delivery of multiple short-harpin RNA (shRNA) targeting not only Tat and Rev transcripts, but also accessory Nef, and major coding regions of Gag, Pol and Env observed the same effect [193].

Earlier studies using TAR RNA “decoy” sequences as competitive inhibitors for Tat recognition could reduce viral replication *in vitro* [194,195]. A phase I clinical trial in four

HIV-1 infected children assessed retrovirus-mediated transduction of a Rev-binding RRE decoy in CD34+ cells isolated from the bone marrow but the treatment was unable to establish multilineage hematopoiesis of cell expressing the Rev inhibitor [196] . Further phase I clinical studies were carried out in HIV-1 infected patients using a Rev-transdominant negative (Rev M10) mutant incorporating two point mutations in highly conserved regions shown that would interfere with Rev function [197]. Autologous CD4+ T cells from HIV-1 infected patients transduced with γ-RVs expressing Rev M10 presented a selective survival advantage, however engraftment efficiency was still limited [198,199].

Besides Tat and Rev, HIV-1 accessory protein Vpr, which also contributes for viral gene expression by arresting cell cycle in G_2 to enhance HIV transcription and translation, was also targeted by gene therapy [160]. Two phase I studies were conducted using CD34+ HSC gammaretroviral delivery of an anti-tat/vpr rybozyme (OZ1), a catalytic RNA that specifically cleaves its target RNA. Long-term survival of CD4+ T-lymphocytes expressing anti tat/rev ribozyme was observed [200], managing to reduce the mean plasma viral load at later time points when it advanced to phase II trial involving 74 patients [201]. This study was the first randomized, double-blind study placebo controlled phase II trial in autologous CD34+ HSCs from HIV patients, proving gene transfer to autologous stem cells is safe and feasible for HIV treatment, but once again the life span and engraftment of modified cells was very limited.

One of the drawbacks of targeting viral pathway is the emergence of resistant strains to gene therapy [202,203]. Combinatorial approach of multiple anti-HIV genes that target distinct steps in the viral replication cycle increase the broad inhibitory potential of these resilient virus [204,205]. An alternative to this approach is targeting endogenous human factors. HIV-1 replication is highly dependent of cell machinery and helper factors [206], thus gene therapy approaches were also designed to target these host proteins essential to viral expression, such as P-TEFb [207] or NF-kB [208,209]. Such approaches demonstrated inhibition of HIV-1 infection *in vitro* but failed to move towards clinical trials. Large-scale screening of helper factors through RNA interference knockdown methods identified over 800 potential host proteins required for viral infection [210–213], still there was very little overlap between these studies and lack of validation of identified targets. Nevertheless, a recent genome-wide screening using a potent gene knockout tool identified and validated three novel targets (TPST2, SLC35B2 and ALCAM) that provided robust resistance to HIV infection consistently in cell lines and primary CD4 T cells across different contexts [214].

Interestingly, non-human factors can also interfere with HIV infection in human cells. Rhesus macaque TRIM5α and owl monkey TRIMCyp were identified as strong HIV xenogeneic restriction factors by targeting and directing viral capsid towards proteasome degradation [215]. Lentiviral delivery of humanized engineered TRIMCyp could potently inhibit HIV viral loads *in vitro* and *in vivo* [216,217], nevertheless potential in treatment of HIV patients still remains elusive. Overall, gene-based therapies to suppress HIV-1 gene expression are promising but still immature at this point. Strategies attempted so far have failed to confer efficient inhibition of HIV-1 replication for a sustained period of time, due to various efficacy and safety issues but also to the emergence of resistant mutants [218].

Ex vivo modification of CD34+ HSCs to render them resistance against HIV-1 entry have been one of the focus of antiviral gene therapy specially since the remarkable case of an unprecedented result achieved in the struggle against HIV-1. In 2009 it was announced the first cure of HIV-1 from an infected patient, which became known as the "Berlin patient". Suffering from acute myelogenous leukemia derived from the HIV-1 infection, this patient was treated with chemotherapy, total body irradiation, and then transplanted with hematopoietic stem cells from a donor homozygous for the CCR5Δ32 mutation [219]. This 32-bp deletion in HIV-1 major cell co-receptor CCR5 gene was found as a natural form of HIV-1 resistance in individuals that were homozygous for this mutation, characterized by the absence of this co-receptor on the cell surface [220,221]. HAART treatment was interrupted one day prior to the transplant, and there is still no sign of any residual HIV-1 viremia up to this date [219,222] However, besides the high risk associated with this action, there is still no evidence if the myeloablative treatment or graft-versus-host effects contributed for the eradication of HIV-1 in this particularly case [219]. In addition, using allogenic (non-self) cells implies locating a bone marrow donor match homozygous to CCR5Δ32 to avoid immune incompatibilities. This is not possible for most individuals, especially considering that the homozygous CCR5Δ32 deletion is only present in 1% of the Caucasian population and even more rare in other ethnic populations [223], making this approach impracticable for standard treatment of HIV-1 infection. In fact, six other patients have received allogenic donor transplant of homozygous *CCR5Δ32/Δ32* HSC but none survived for longer than one year [224], and in one case it was detected a shift in HIV tropism from CCR5 to CXCR4 [225]. Nevertheless, this notable case proved that it was possible to eliminate HIV infection by infusing the patient with resistant cells that lacked the virus co-receptor.

Even prior to this achievement, researchers had already attempted to produce the CCR5 deletion on autologous (self) cells before reinfused back into the patient, avoiding issues with immunological incompatibility observed with allogenic cell transfer [168]. Developed gene therapeutic strategies to delete CCR5 surface expression used lentivirus-delivery of shRNA [226–228] or ribozymes [229,230] that targeted CCR5 transcription, or CCR5-specific single-chain intrabodies that retained the co-receptor in the endoplasmic reticulum preventing the co-receptor emergence to cell surface [231,232]. Overall, CCR5 inhibition generated HIV resistant CD4+ T cells protected from CCR5-tropic virus infection *in vitro* and *in vivo*. Furthermore, human CD34+ HSCs transduced *ex vivo* with a lentivirus incorporating a CCR5 ribozyme along with an anti-tat/rev shRNA and a TAR decoy also generated HIV resistant monocytes and thymocytes [233,234]. This combinatorial approach led to a phase II clinical trial in AIDS lymphoma patients transplanted with autologous transduced HSCs [235]. Follow up indicated that less than 0.2% of modified cells were detected in peripheral blood after one year and no immediate clinical benefit was observed.

In consonance with gene therapy targeting HIV replication, these approaches were often unable to sustain CCR5 knockdown. Strong expression of the anti-CCR5 genes must be maintained for long-term often resulting in cytotoxicity, especially with anti-HIV shRNA that can interfere with the endogenous microRNA pathway [204,236,237]. Genome engineering through site-directed nucleases have emerged as a prominent alternative to knockout CCR5 in an irreversible and more efficient manner.

1.2.3. Genome engineering

Traditionally, gene therapy counters genetic-associated diseases by inserting a functional copy of the symptom-associated gene. Although promising, this concept is not free of challenges towards implementation. In general, long-term stable expression of therapeutic genes require genome integration into replicating cells, which can have unpredictable effects on gene expression and also cause toxicity derived from random integration [238]. Moreover, much of these integrated genes are regulated by constitutive exogenous promoters that do not always reflect the normal gene regulation in physiologic context-dependent. In addition, some of these therapeutic genes are too large to be packaged into available delivery vectors. Finally, addition of exogenous genes is unable to address the presence of toxic or unwanted gene products that directly or indirectly promote the pathogenic disease, such as viral genomes or receptors [239].

The prospect of directly target and correct the defective or unwanted gene instead of just adding an exogenous copy paved the way for the rise of genome engineering. Over the quest of correcting defective genes associated with severe pathologies, genome engineering is the ultimate revolution in biomedical research and regenerative medicine. Development of cutting-edge technologies have enabled the generation of "surgical scalpels" that can target and make precise modifications in a small DNA region among billions of base pairs that constitute the human genome. These tools are based on DNA-binding domains that naturally exist in life forms but were found to be programmable and repurposed to target any DNA sequence in human cells through synthetic biology.

Three major DNA-binding domains (DBD) have been widely adopted for genome engineering applications: zinc-fingers (ZF), transcription activator-like effectors (TALEs) and CRISPR-Cas9 system (Fig. 1.18). Each of these targeted domains have a particular DNA recognition mode that is engineered for targeting the desired sequence and additionally, their DNA-binding domain is completely separated from the natural catalytic domain [240]. Their versatility allows researchers to assemble a wide panel of genome modification effector domains to attain distinct outputs [239–241].

Zinc-Finger [242,243] and TALE [244,245] proteins are programmable DNA-binding domains derived from the assembly of modules that mediate interaction between amino acid side chains of the DBD and the nucleotides of the target sequence. However, the recent discovery that the bacterial adaptive immunity CRISPR-Cas9 system [246] could be redirected to perform human genome engineering have revolutionized this field. Opposed to zinc-finger and TALE proteins, Cas9 protein is directed to the target sequence by a small guide RNA (gRNA) that mediates binding to the complementary DNA strand via Watson-Crick base-pairing. The small size of the gRNA targeting domain (20 nucleotides) permit the quick synthesis of these oligonucleotides to direct the Cas9 to a unique or multiple DNA sites without the need of protein engineering [246]. This unique feature has greatly simplified DNA targeting [247–250] and led to the general adoption of this system for a wide range of genome engineering applications [251].

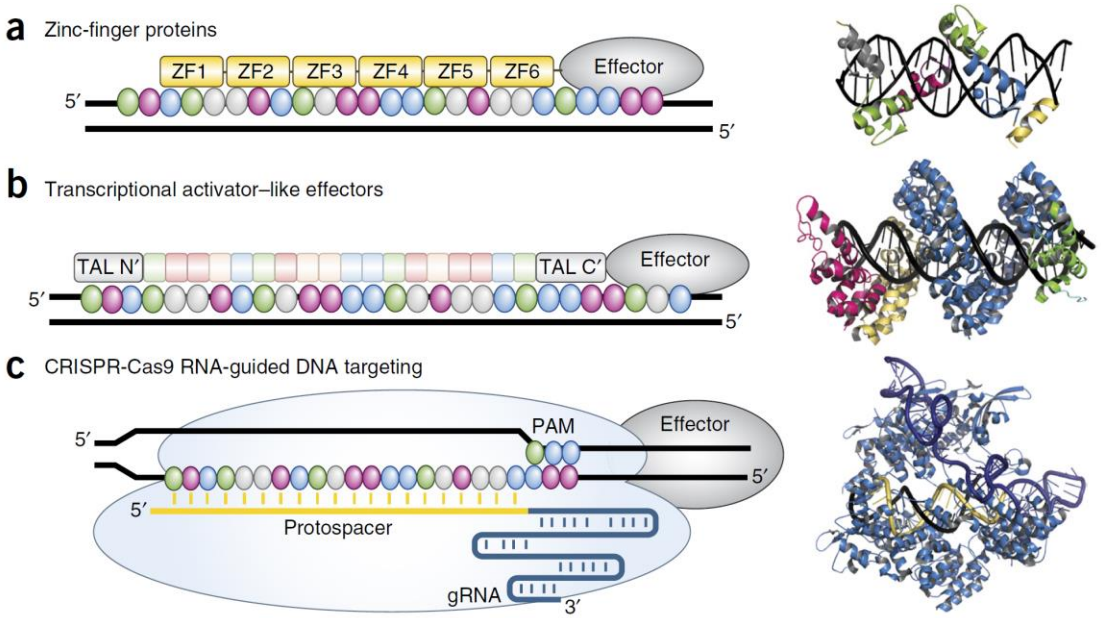


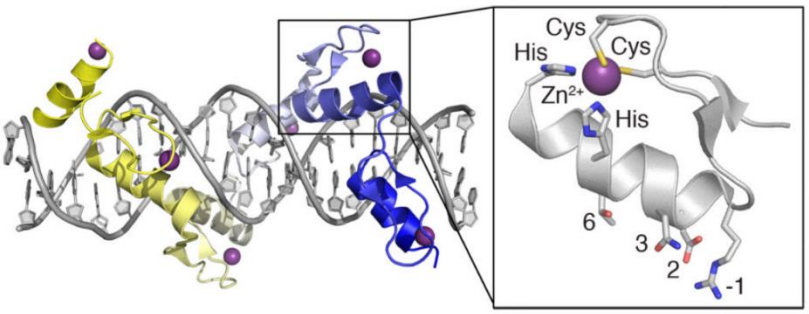
Figure 1.18- Programmable DNA-binding domains for genome engineering applications. Schematic representation of DNA targeted platforms (left) and corresponding crystal structure (right). a) Zinc-finger proteins are assembled by modules that each recognize 3-4 nucleotides. **b)** Transcription activator-like effectors (TALEs) DNA-targeting domain is located at the central region with a composition of array of modules, each recognizing a single nucleotide. **c)** CRISPR-Cas9 is directed to the DNA target by a guide RNA (gRNA) consisted of a 20-nucleotide sequence (protospacer) complementary to the genomic sequence via Watson-Crick base-paring and a constant region that interacts with the Cas9 protein. The DNA-target sequence is located next to a protospacer adjacent motif (PAM), required to promote Cas9 binding (5'-NGG-3' for Cas9 derived from *S. pyogenes*). Source: *Thakore et al. 2016* [241].

DNA-targeting of Cas9 by the gRNA complimentary sequence (protospacer) is restricted however to the presence of a protospacer adjacent motif (PAM) at the 3' end of the target sequence. The PAM motif varies according to the Cas9 strain used (5'-NGG-3' for the most common Cas9 strain derived from *S. pyogenes*) [252]. The requirement for PAM limits the targeting range of Cas9.

In the next chapter, we will focus on structural and functional aspects of genome engineering tools implemented in this dissertation: Zinc-fingers and TALE binding domains.

1.2.3.1. Cys2His2 Zinc-finger proteins

Zinc-finger proteins were the first engineered platforms adopted for gene targeting in human cells, due to their modularity and their capability to bind DNA in a specific and efficient manner [242]. The Cys2His2 motif is the best characterized zinc-finger domain, standing among the most frequent DNA-binding domains in eukaryotic cells. Structural studies have demonstrated that an individual zinc-finger domain consists of approximately 30 amino acids arranged in a conserved ββα arrange. This conformation is folded by a zinc ion coordinated by two cysteine and two histidine residues [253]. The α-helix typically contacts 3 base-pairs (bp) or triplets with DNA-binding residues located at the position -1, 3 and 6, critical to determine specificity towards the major groove of the DNA sequence (Fig. 1.19; Top). Nevertheless, residues at positions 2, 4 and 5 are also important to enforce target specificity and minimize off-target towards other triplets [243]. The zinc-finger domain is positioned backwards relatively to the DNA strand, meaning the N-terminal finger contacts the 3'-terminal DNA triplet, and so on. Through phage display technology, Barbas and colleagues screened for zinc-finger domains that bound most of the possible 64 triplets through randomization of the α-helix residues. In these studies, synthetic zinc-finger domains were identified that bound all possible 16 5'-GNN-3' [254,255], 5'-ANN-3' [256], and 5'-CNN-3' triplets as well as some 5'-TNN-3' targets [257]. The work established by Carlos Barbas was crucial for the understanding of zinc-fingers interaction with target DNA and the establishment of predetermined codes between the sequence targeted and the corresponding zinc-finger optimal α-helix domain [243] (Fig. 1.19; Bottom).



Second position

First position	T	C	A	G	Third position
T	TTT -	TCT - RLRDIQF	TAT - ARGNLRT	TGT -	T
	TTC -	TCC - RSDERKR	TAC - SRGNLKS	TGC - APKALGW	C
	TTA -	TCA - RSDHLTT	TAA - QASNLIS	TGA - QAGHLAS	A
	TTG - RKDALRG	TCG - RLRALDR	TAG - REDNLHT	TGG - RSDHLTT	G
C	CTT - TTGALTE	CCT - TKNSLTE	CAT - TSGNLTE	CGT - SRRTCRA	T
	CTC - QRHHLVE	CCC - SKKHLAE	CAC - SKKALTE	CGC - HTGHLLE	C
	CTA - QNSTLTE	CCA - TSHSLTE	CAA - QSGNLTE	CGA - QSGHLTE	A
	CTG - RNDALTE	CCG - RNDTLTE	CAG - RADNLTE	CGG - RSDKLTE	G
A	ATT - HKNALQN	ACT - THLDLIR	AAT - TTGNLTV	AGT - HRTTLTN	T
	ATC - RRSACRR	ACC - DKKDLTR	AAC - DSGNLRV	AGC - ERSHLRE	C
	ATA - QKSSLIA	ACA - SPADLTR	AAA - QRANLRA	AGA - QLAHLRA	A
	ATG - RRDELNV	ACG - RTDTLRD	AAG - RKDNLKN	AGG - RSDHLTN	G
G	GTT - TSGSLVR	GCT - TSGELVR	GAT - TSGNLVR	GGT - TSGHLVR	T
	GTC - DPGALVR	GCC - DCRDLAR	GAC - DPGNLVR	GGC - DPGHLVR	C
	GTA - QSSSLVR	GCA - QSGDLRR	GAA - QSSNLVR	GGA - QRAHLER	A
	GTG - RSDELVR	GCG - RSDDLVR	GAG - RSDNLVR	GGG - RSDKLVR	G

Figure 1.19- Structure of zinc-finger domains. (Top) Representation of the Cys2His2 zinc-finger motif bound to target DNA (grey). Each zinc-finger domain consists of approximately 30 amino acids in a ββα arrangement. The α-helix and antiparallel β-sheet are folded by a zinc ion (purple) coordinated by two cysteine and two histidine residues, depicted as sticks. The α-helix surface residues (-1, 2, 3 and 6) that promote DNA contact are illustrated as sticks. Source: *Gaj et al. 2013* [240]. **(Bottom)** Summary of the zinc-finger domains used for each target, represented by the α-helical amino acids selected. The α-helix residues (-1, 3 and 6) that contact the major groove of the DNA strand are underlined. Source: *Gersbach et al. 2014* [243].

The modular structure of zinc-fingers have opened many opportunities for drug discovery and gene therapy based approaches [258]. Design of zinc-finger proteins binding 18 bp can confer unique specificity within more than 3 billion bp of the human genome [259], allowing for the first time targeting specific sequences for genome engineering applications in human cells [254,260].

Having deciphered the code for zinc-finger domains to target specific DNA sequences and the means to assemble them to achieve genome-wide specificity, a vast number of genome engineering applications have arisen from zinc-finger technology. Due to their versatility, zinc-fingers were assembled to effector domains and repurposed for a variety of applications, ranging from generation of artificial transcription factors or chromatin modulators to targeted

nucleases and recombinases that would target genes associated with human disorders (Fig. 1.20) [243] or even create zinc-finger libraries to find potential genes that control tumor progression [261] or generate drug resistance [262].

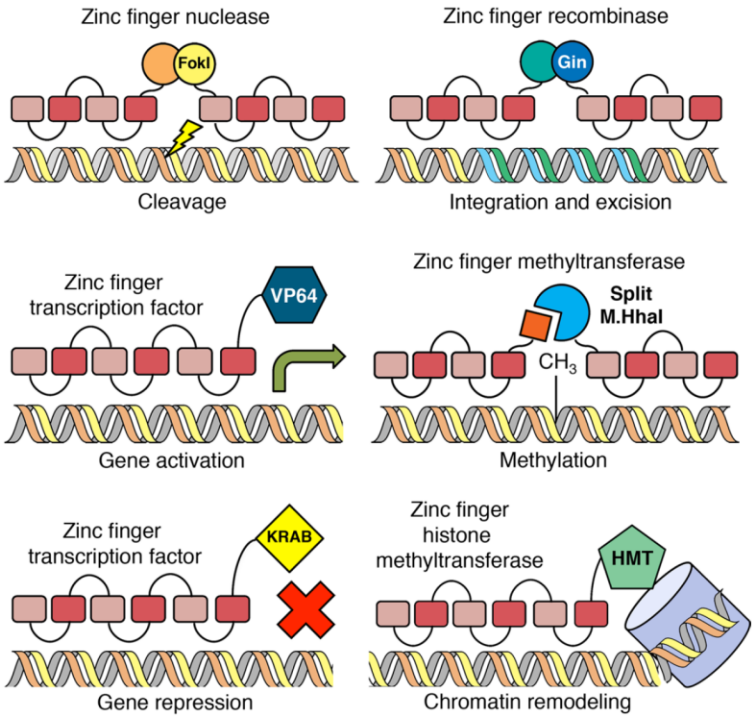


Figure 1.20- Modular assembly of DNA-binding zinc-fingers with effector domains for precise modifications in the human genome. Source: *Gersbach et al. 2014* [243].

Standard zinc-finger delivery methods present limitations such as toxicity derived from plasmid transfection or electroporation, viral vectors immunogenicity, and potential for mutagenic effects derived from vector integration into the genome. Due to the net positive charge involving zinc-finger proteins, these were found to possess the innate ability to cross the anionic cellular membrane [263]. Purified zinc-finger nuclease (ZFN) proteins are able to penetrate a wide variety of mammalian cells, including primary CD4+ T cells or CD34+ HSCs, and perform targeted gene editing *ex vivo* without the need for additional protein engineering [263,264]. This remarkable property confers an advantage to ZFN-mediated gene editing since delivered protein is shortly degraded resulting in less off-target activity while avoiding DNA insertional mutagenesis [263,264].

Overall, zinc-finger technology has pioneered the application of genome engineering for human diseases and the progress obtained with these proteins contributed to thrive researchers to pursuit novel DNA-binding and expand the genome engineering toolbox.

1.2.3.2. Transcription activator-like effectors

In 2009, the decipher of DNA targeting mode of transcription activator-like effector (TALE) proteins from the plant pathogenic bacteria *Xanthomonas sp.*, has enthused researchers about the prospect of a novel genome engineering tool with a more straightforward modular recognition of DNA. Opposed to the zinc-finger proteins, transcription activator-like effectors (TALE) possess a simpler DNA recognition code [244,245]. TALEs central region is composed of an array of DNA recognition domains of ~34 amino acid repeats that each recognizes a single DNA bp. The TALE specificity for each bp is mediated by two variable residues at position 12 and 13 named repeat-variable diresidues (RVD) (Fig. 1.21). Conjugations of several RVD domains targeting single bp generate site-directed TALEs able to recognize contiguous DNA sequences of 15-40 bp without the need of linker adjustment [265]. The TALE N-terminal domain recognizes the 5' terminal nucleotide (N_0) at the binding start site. General guidelines suggest that its activity is optimized when the binding site starts with a T base [266,267]. Nevertheless, direct evolution of N-terminal domain generated TALEs with optimal recognition of all 5' base-pairs [267].

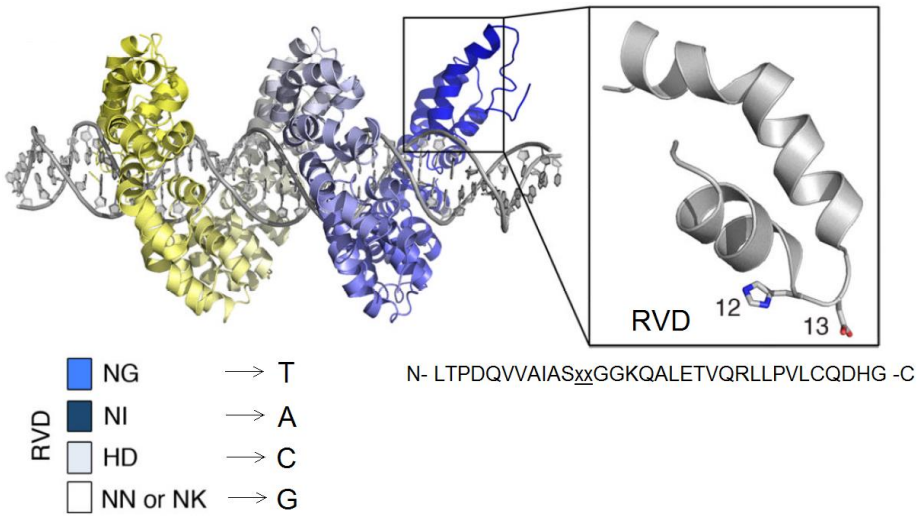


Figure 1.21- Structure of transcription activator-like effectors. Representation of the TALE protein bound to target DNA (grey). Individual TALE repeats consist in 33-35 conserved amino acids except at positions 12 and 13. These two hypervariable residues (shown as sticks) mediate recognition of a single base-pair and are known as repeat-variable diresidues (RVD). Sequence of TALE repeat is indicated below its structure with RVD position underlined. Specificity towards different base-pairs varies according to the RVD compositions indicated. Adapted from *Gaj et al. 2013* [240].

Based on the RVD-bp one-to-one simplicity of TALE assembly to target any DNA sequence in the human genome, numerous TALE effectors have been designed for genome engineering applications such as transcription activators [268,269] or repressors [270], site-directed nucleases [268,271,272], recombinases [273] and epigenetic effectors [274–276]. The ease by which TALE arrays can be assembled using the Golden Gate method enabled the construction of libraries targeting a large range of human genes [277] that facilitated genome-wide screening studies for identification of novel targets to counter human disorders. As a result, TALEs have achieved widespread use throughout biotechnology, with the potential to impact future developments in human gene therapy.

1.2.3.3. Genome engineering applications for gene therapy

In general, genome engineering applications focus on gene editing of the human genome [239,251]. Gene editing is performed when targeted nucleases based on the DNA-binding domains promote double-strand breaks (DSB). When repaired, DSB can generate random insertions or deletions introduced by the non-homologous end joining (NHEJ) pathway that knockout (or eventually restore) target genes by frameshifting, or alternatively cause large gene deletions or chromosome translocations after promoting DSB at two distal sites. In the presence of a donor template with homologous sequences to the target site, DSB can be resolved by homology-directed repair (HDR) that can directly correct the defective gene or promote gene integration into a specific locus (Fig. 1.22; Left). Another side of the genome engineering is modulation of gene transcription, either through genesis of artificial transcription factors that directly activate or repress target genes, or by modulating chromatin marks such as histone acetylation or DNA methylation through epigenome editing [241] (Fig. 1.22; Right).

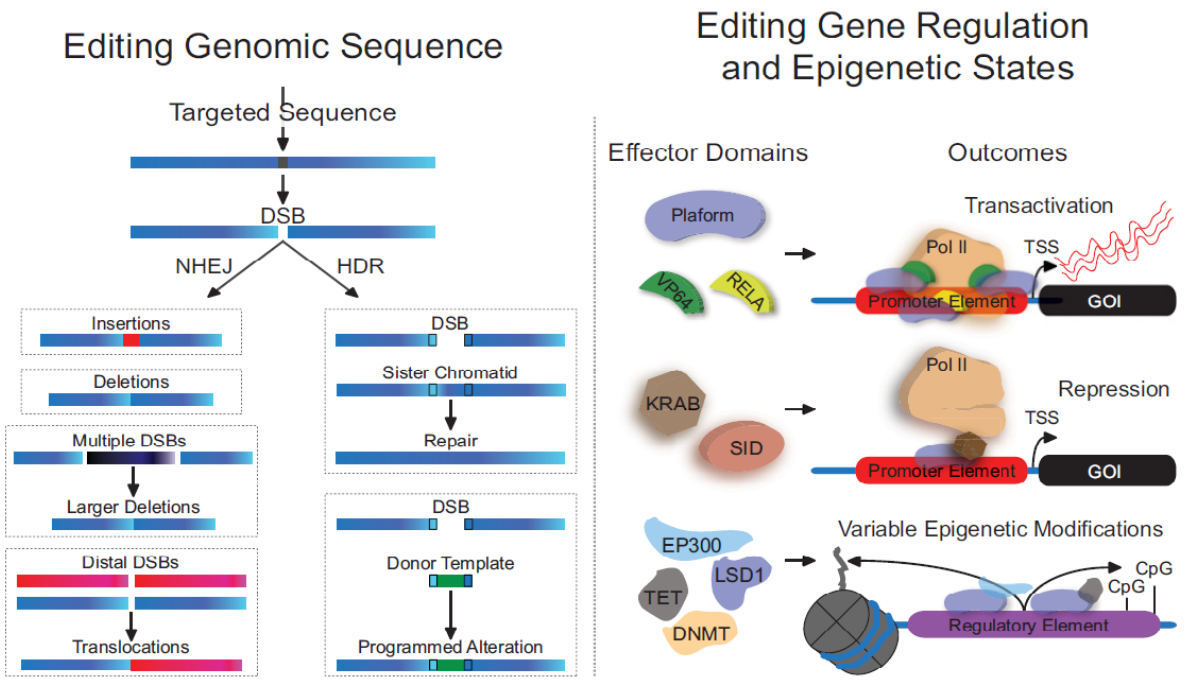


Figure 1.22- Precise modifications in the human genome by genome engineering. (Left) Gene editing of genomic sequence. DNA-binding domains when fused to nuclease catalytic domains can promote targeted double-strand breaks (DSB) in the human genome. DSB can be repaired by non-homologous end joining (NHEJ) through random nucleotide insertion or deletion leading to frameshifts that disrupt the gene ORF. In addition, large deletions can also be performed and repaired by NHEJ when two targeted nucleases cut at distal sites. Alternatively, DSB can be repaired by homology-directed repair (HDR) in the presence of a donor template that share homologous sequences with the adjacent regions of the DSB. HDR can be used for site-specific gene correction or single base pair repair or alternatively, targeted integration of a full-length therapeutic gene. **(Right)** Epigenome editing and transcriptional regulation. Targeted DNA-binding domains can be fused with effector domains that modify and regulate gene expression. These artificial transcription factors can either activate or shutdown a gene of interest (GOI) directly by fusing to activators or repressors of gene transcription or by modulating chromatin epigenetic marks such as DNA methylation or histone acetylation. Source: *Hilton and Gersbach 2015* [278].

Opposed to traditional gene therapy, genome engineering does not require sustained gene expression to achieve clinical benefit. Transient expression of site-directed DBD is sufficient to modify the defective genomic trait through "hit and run" strategies that enable genome modification but minimizes exposure to DBD off-target activity [239,279]. The most used method for delivery of genome engineering proteins is through plasmid DNA electroporation, an easy and fast method to promote high levels of gene expression but reveals a number of issues such as DNA-related toxicity, low efficiency in primary cells or other types, and potential for recombination of plasmid within the cell genome [280,281]. Instead, mRNA electroporation overcame some of these advantages while retaining high efficient gene

expression, becoming a preferred method for *ex vivo* gene editing of HSCs [248,282]. Direct protein delivery through electroporation [283,284] or engineering of cell-penetrating nucleases [263,285–287] have surprisingly achieved high gene editing levels *ex vivo* with minimal toxicity.

Viral vectors are highly desirable due to efficient and wide range of cell transduction and are optimal vehicles to deliver exogenous templates for HDR gene correction or integration [139]. Non-integrative lentivirus can efficiently transduce primary T cells and HSCs without causing undesired vector integration [138] and therefore have been widely used for transient nuclease delivery or as HDR substrates for *ex vivo* gene editing [156,288]. Adenovirus have likewise been successful in providing transient nuclease expression for *ex vivo* gene editing [289], also benefiting from high efficiency transduction in a variety of cell types and large packaging capacity to deliver sizable cassettes, being generally preferred over lentiviral methods for TALE nuclease editing [290,291]. Recombinant adeno-associated virus or rAAVs are highly promising for genome engineering *in vivo* due to their broad gene delivery potential, low immunogenicity and innate ability to promote homologous recombination [109], achieving exciting results when targeted to mouse liver [292,293], brain [294–296] or muscle [297–299]. Nevertheless, their limited packaging capacity limits their ability to carry large TALE or Cas9 nucleases or HDR substrates.

In addition to biomedical application, genome engineering benefits can extend to other broad applications from basic research to biotechnology, including but not limited to: facilitate the generation of cellular or animal models; provide useful synthetic materials or sustained biofuels derived from engineered microorganisms; improve agriculture crops resistance to severe environment or pathogenic infection; or enhance bacterial production of therapeutic drugs to reduce cost and accessibilty to such pharmaceuticals [300].

1.2.3.3.1. Transcriptional modulation and epigenome editing

In several pathologies, therapeutic benefit can be achieved by silencing or activating the target gene rather than modifying its sequence. Transcriptional modulation or epigenome editing through artificial transcription factors that targets a gene promoter or regulatory element has offered an alternative solution to silence or restore target gene expression without causing disruptive breaks in the double-helix DNA strand. Zinc-fingers [301] and TALE [302] modular proteins can be assembled to effector domains that regulates gene transcription (Figure 24; right). CRISPR-Cas9 is a natural endonuclease, however inactivating mutations in the catalytic

domains RuvC and HNH generates a deactivated Cas9 (dCas9) that is unable to cut DNA but retains its DNA recognition ability [303], allowing this system to be repurposed for modulation of gene transcription in a similar way to ZF and TALEs.

Earliest reports of programmable transcription activators fused zinc-fingers to VP16, a Herpes viral activation domain that recruits PolII transcriptional machinery to induce gene activation [259,304]. The generation of VP64 [301], a synthetic tetramer of VP16, substantially increased activation potency and has been widely adopted for generation of transcription activators [241]. Previous reports demonstrated that recruitment of multiple TALE or dCas9 activators to a single locus synergistically enhanced gene activation [302,305–310]. In addition, natural transcription activation is characterized by multiple recruitment of distinct transcription factors and chromatin remodelling complexes [311]. This concept led to the exploitation of strategies to provide robust gene expression with a single activator that recruit multiple activation domains, ultimately culminating in the development of next-generation activators such as VPR (a tripartite fusion of VP64, RTA and p65 activation domains) [312], SunTag (antibody-mediated tagging of multiple VP64) [313] or synergistic activaton mediator (SAM; dCas9-VP64 fusion with gRNA-recruitment of p65 and HSF1 co-activators) [314]. These methods outperformed by far the standard VP64 activator in multiple context and organisms [315], being able to promote sustained gene expression and potentiating genome-wide gene activation screens [314].

Targeted gene reppression through site-directed DBDs emerged as an alternative to RNA interference, which presents limitation towards sustained gene silecing, off-target effects and toxicity associated with oversaturation of endogenous microRNA pathways [316,317]. Furthermore, programmable DBD can target and silence regulatory elements and non-coding regions that RNAi cannot. The most common reppressor domain used for site-directed gene silencing is Krüppel-associated box (KRAB) which silence transcription by recruiting multiple histone methyltransferases and deacetylases [318–321], being able to shut down a wide range of target genes when fused to DNA-binding domains [254,270,322].

Instead of directly modulating gene expression by recruiting transcription factors, it is possible to do so by remodelling the chromatin epigenetic marks sorrounding to the target gene, a strategy known as epigenome editing. Epigenome edting is particularly desired due to the long-term and inheritable gene modulation which can persist through cell replication [241]. Epigenetic effectors located to a specific locus can catalyze modifications to DNA methylation or histone acetylation/methylation status to activate or silence a target gene [241,323,324]. TET

and TGD epigenetic effectors can promote transcriptional activation through CpGs demethylation at endogenous promoters when directed by ZF or TALE proteins [274,325,326]. Alternatively, DBDs can be fused to catalytic core of p300 histone acetyltransferase that remodel chromatin compactation to activate promoter regions unresponsive to transcription factors or even distal enhancers [276]. Reversal of these modifications by targeted reppressive DNA methyltransferases (e.g. DNMT3a) or histone deacetylases (e.g. LSD1) have shown to reppress transcription from endogenous promoters or active enhancers when tethered by ZF, TALE or CRISPR-dCas9 domains [327–330]. Transient expression of targeted DNA methyltransferases have shown to permanently induce gene silecing that can only be reversed by targeted demethylation effectors [331,332].

Methods to treat human disease through transcription regulation or epigenome editing are generally applied in cases where abnormal gene regulation is the causative factor of the disorder [333]. Site-directed transcription factors or epigenetic modulators provide the opportunity to restore normal gene regulation, up-regulate genes that counter the symptomatic effect or silence genes/pathways that are reponsible for the disease progression.

Hematological disorders such as sickle cell anemia or β-thalassemia, caused by disruption of β-globin expression can be countered by activation of γ-globin through ZF or dCas9 engineered activators [308,334,335]. TALE-VP64 activators have demonstrated potential to treat Friedreich's ataxia, a neurodegenerative and cardiac disroder caused by loss of frataxin gene expression [336,337]. Activation of endogenous vascular endothelial growth factor (VEGF) is pursued to enhance wound healing or generate neovasculature in patients with diabetic neuropathy and peripheral arterial disease. Engineered ZF activators targeting VEGF promoter were able to activate all isoforms of this growth factor and stimulate angiogenesis expression and neovascular formation *in vivo* [338,339]. On the other hand, silencing VEGF expression through ZF targeted reppression [340] or methylation [328] is able to reduce tumour angiogenesis. Other ZF reppressors were developed to target oncogenes and inhibit cancer progression [341,342]. Other disorders targeted by ZF reppressors include neutodegenerative Huntington's disease [296] or HIV infection (detailed in chapter 1.2.2.3) [343–346].

In addition to treat human disorders, targeted transcription modification or epigenome editing is an important asset to interrogate gene function and correlation with chromatin state and cell phenotye for a wide range of applications (Fig. 1.23) [241].

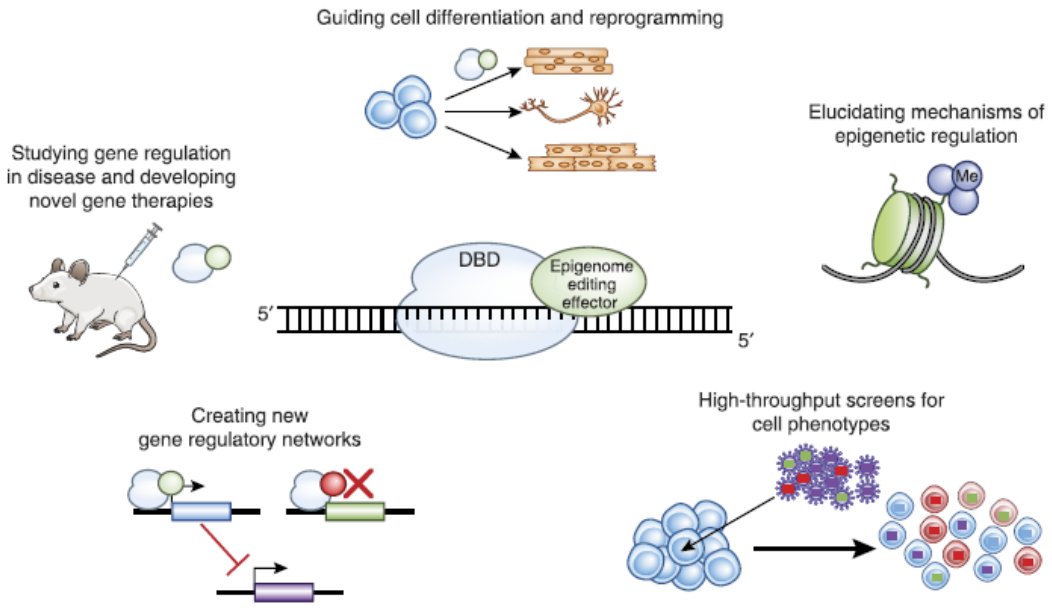


Figure 1.23- Diversity of epigenome editing applications. Source: *Thakore et al. 2016* [241].

Considering that a vast number of diseases is caused by complex genetic variations, knowledge obtained from gene or chromatin interrogation could provide novel approaches to treat human disease. Study of gene regulation is challenged by the existence of multiple putative elements that often regulate gene expression at a distance. Epigenetic modulators can target distal enhancers to reveal their role in the human genome context, enabling modulation of multiple genes with a single epigenetic editing protein [276,347]. Design or modulation of complex gene networks can elucidate mechanisms controlling cellular programs such as cell migration, tissue development or inflammatory response [241]. For this purpose, targeted orthogonal systems that can promote different epigenetic outputs at distinct loci have tremendous potential. The CRISPR-Cas9 system versatility enables simultaneous gene activation and reppression [348] or even knockout [349,350] at different target genes using the same Cas9 protein in a programmable manner by engineering the gRNA to recruit different effector domains according to the target site.

Development of site-directed transcription factors [314,351–353] libraries that target and control expression of all potential human genes brought great impact for screening and identification of genes responsible for induction of signaling pathways, disease progression or resistance and cell programming. Finally, targeted epigenetic modulators could represent an effective tool to guide cell differentiation or reprogramming back to pluripotency, a proeminent method of cell-based therapy for regenerative medicine. Site-directed TALE or dCas9 gene modulators that control expression of key transcription factors critical to determine cell fate

were able to reprogram and generate relevant cell types from induced pluripotent stem cells to neurons or skeletal myocites [312,354,355]. In conclusion, modulation of transcripional or epigenome marks in a tunable and reversible manner by these evolved programmable enzymes promises to bring new insights on the complex mechanisms involving gene regulation and cell programming for the development of improved gene and cell-based therapies.

1.2.3.3.2. Targeting HIV-1 infection through genome engineering

Genome engineering applications were specially focused at correcting hereditary gene defective pathologies. Nevertheless, it didn't take long before researchers expanded their attention to develop strategies to counter acquired diseases such as antiviral, with HIV naturally being at the center of these approaches. The reported case of the "Berlin patient", cleared of HIV for almost 10 years after transplantation with HIV-resistant HSC homozygous for CCR5Δ32 mutation (see chapter 1.2.1.3) [219], had motivated researchers to keep pursuing therapeutic approaches that replicate this effect. Efforts to reproduce the CCR5 deletion were mostly based on gene therapy that either shut down CCR5 transcription using RNA interference methods [226–230] or prevent CCR5 emergence to the cell surface [231,232]. However, none of these reports have shown reduction in patient viremia, being associated with ineffective long-term CCR5 inhibition and toxicity derived from requirement of sustained high levels of transgene expression [192]. These limitations showed that these methods were still far from being evolved to the point of providing clinical benefit.

The development of targeted nucleases for gene editing may have provided the solution to efficiently eliminate CCR5 co-receptor permanently and turn cells resistant to HIV-1 entry with a single treatment [356] (Fig. 1.24). Disruption of *CCR5* gene through zinc-finger nucleases has proven to reduce HIV-1 viremia in CD4+ T cells [289] and HSCs [357] *in vitro* and *in vivo* in mouse models of HIV-1 infection engrafted with modified resistant cells. Promising results led to the first clinical trial of ZFN-mediated gene editing in a phase I study involving 12 patients from which autologous CD4+ T cells were transduced *ex vivo* with an adenoviral vector expressing a CCR5-ZFN and re-infused in the patient [358]. Preliminary results demonstrated that CCR5-modified autologous CD4+ T cells were safe within this study.

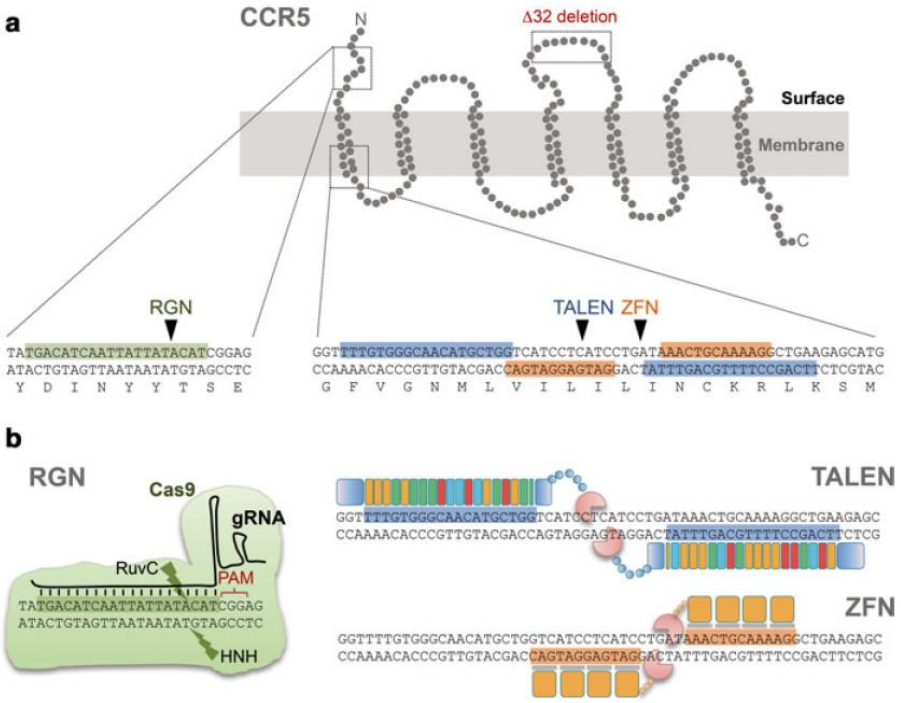


Figure 1.24- Targeted nucleases designed to disrupt CCR5. a) Schematic representation of the CCR5 co-receptor located at the cellular membrane. Dotted boxes indicate the region targeted by engineered nucleases and the Δ32 deletion. Three targeted nucleases were design to efficiently knock out *CCR5*. Corresponding DNA target sites are highlighted in green for Cas9 RNA-guide nuclease (RGN), blue for TALEN and orange for ZFN. Cleavage sites are pointed out by black triangles. **b)** Schematic representation of the designed nucleases targeting *CCR5*. RGN cut the CCR5 gene through their nuclease domains within Cas9 protein (RuvC and HNH). TALEN and ZFN promote DSB at *CCR5* by assembling the modular DNA-binding domain with the FokI nuclease domain (light red), which cuts the DNA upon dimerization of the two monomers at the target site. Source: *Cornu et al. 2015* [356].

The advancements made with this approach had such potential that completed and ongoing gene editing clinical trials are dominated by ZFN-mediated deletion of *CCR5* [239] (Table 1.1). Two studies however revealed a significant level of genome wide off-target by CCR5-ZFN [359,360], raising concerns about the use of this platform for HIV treatment. Considering their straightforward modular DNA recognition, TALENs were able to knock out CCR5 with similar activity and lower toxicity [272,361], being a valid alternative to generate HIV resistant cells. Naturally, further studies to disrupt *CCR5* were pursued using RNA-guided CRISPR-Cas9, a suitable platform for multiplex targeting [250], also achieving promising results [248].

Table 1.1- Representative completed and ongoing gene editing clinical trials.

Source: *Maeder and Gersbach et al. 2016* [239].

Identifier	Phase	Title	Status as of October 2015
NCT00842634	Phase 1	Autologous T Cells Genetically Modified at the CCR5 Gene by Zinc Finger Nucleases SB-728 for HIV	Completed
NCT01044654	Phase 1	Phase 1 Dose Escalation Study of Autologous T Cells Genetically Modified at the CCR5 Gene by Zinc Finger Nucleases in HIV-Infected Patients	Completed
NCT01082926	Phase 1	Phase I Study of Cellular Immunotherapy for Recurrent/Refractory Malignant Glioma Using Intratumoral Infusions of GRm13Z40-2, An Allogeneic CD8+ Cytolitic T Cell Line Genetically Modified to Express the IL 13-Zetakine and HyTK and to be Resistant to Glucocorticoids, in Combination With Interleukin-2	Completed
NCT01252641	Phase 1/2	Study of Autologous T Cells Genetically Modified at the CCR5 Gene by Zinc Finger Nucleases in HIV-Infected Subjects	Completed
NCT02225665	Phase 1/2	Repeat Doses of SB-728mR-T After Cyclophosphamide Conditioning in HIV-Infected Subjects on HAART	Active
NCT01543152	Phase 1/2	Dose Escalation Study of Cyclophosphamide in HIV-Infected Subjects on HAART Receiving SB-728-T	Recruiting
NCT02500849	Phase 1	Safety Study of Zinc Finger Nuclease CCR5-modified Hematopoietic Stem/Progenitor Cells in HIV-1 Infected Patients	Recruiting

While gene editing of *CCR5* enables modified cells to survive and expand in the presence of R5-tropic strains, these cells could still be susceptible to CXCR4-strains. Once HAART is discontinued, X4-tropic strains could emerge and lead to viral rebound. In order to avoid this potential hazard, simultaneous disruption of *CCR5* and *CXCR4* by ZFN were reported to protect CD4+ T cells from both R5 and X4-tropic strains in a mouse model [362]. However, opposed to CCR5, CXCR4 seems to have a critical role in immune regulation, particularly in B cell development [363]. This limitation narrows the possibilities of CXCR4 gene editing in HSCs, restricting this application to CD4+T cells only [362,364], that in comparison has far less long-term clinical benefit [365].

Genome engineering to turn cells resistant to HIV also extended to host restriction factors by targeting Lens epithelium-derived growth factor (LEGDF/p75) encoded by the *PSIP1* gene. LEDGF/p75 binds HIV integrase, acting as a tether domain to facilitate integration of provirus cDNA into host genome [366,367]. Disruption of *PSIP1* by ZFN [368] or TALEN [369] reduced HIV inhibited in cell lines. Nevertheless, LEDGF/p75 knockout in mice caused prenatal death or development of abnormalities, questioning the viability of LEDGF as a potential target for HIV treatment [370].

Besides engineering HIV resistant cells, targeted nucleases have also been developed to directly target and disrupt the HIV provirus. This strategy implies the direct delivery *in vivo* of designed nucleases to the infected cells, without the need for autologous cell manipulation and transplant. Another advantage of this approach is that in most cases only one copy of integrated provirus needs to be inactivated, as opposed to the two copies of host genes of the diploid genome [371]. ZFN [372], TALEN [373] and CRISPR/Cas9 [374,375] nucleases, most

targeting the LTR regions flanking the provirus, have demonstrated to efficiently excise HIV genome and reduce viral loads *in vitro*, both on active and latent infected cells. Similar to its natural adaptive immune role in bacteria, CRISPR-Cas9 nucleases can also be harnessed for antiviral response in human cells. In addition to target and excise HIV provirus from already infected cells, stably expressing CRIPR/Cas9 can also be used as an intracellular defense system against HIV-1 by targeting and inactivating pre-intregrated dsDNA, protecting the cells from novel infections [376]. Surgical removal of HIV from infected cells using engineered nucleases might raise some concerns about the variability introduced into the viral genome by NHEJ repair. Recent reports have demonstrated the emergence of treatment resistant HIV strains presenting random insertions or deletions [377–381], pointing out that extreme caution should be taken when targeting nucleases to disrupt HIV genes.

Prior to the development of targeted nucleases against HIV, application of evolved recombinases also demonstrated exciting results in the excision of HIV genome. In 2007, Hauber and colleagues developed a Tre recombinase (HIV LTR-recombinase) by direct evolution of wild type Cre recombinase [382] that shifted the natural recognition of *loxP* site to an assymetric sequence within the HIV LTR region [383]. Site-specific recombinases catalyze DNA rearrangements, removing genomic sequences while avoiding the NHEJ-mediated random variability [382]. Tre recombinase efficiently excised the HIV-1 proviral genome from infected cells [383]. More recently, these authors further evolved Cre to a broad HIV-recombinase (Brec1) that recognizes more conserved regions in the LTR. Brec1 precisely and safely removed integrated HIV from various clinical isolates *in vitro* and *in vivo*, including mice humanized with patient-derived cells [384].

Alternatively to gene editing, programmable DNA-binding platforms have been associated with effector domains that regulate gene transcription or modulate chromatin epigenetic marks [241] (for more details see chapter 1.2.3.3.2.). Instead of using RNA interference methods [192], researchers have successfully developed zinc-finger transcription repressors KRAB-ZF that directly target LTR promoter and shutdown HIV transcription while reducing viral loads [343–346]. While RNAi must inactivate several mRNA copies which may difficult complete and sustained repression, this strategy has the advantage to target only one copy of the HIV provirus.

Even in the absence of effector domains, DNA-binding proteins can still inhibit HIV replication. One example of this is the development of zinc-fingers that target the formation of 2-LTR conformation of the pre-integration complex, formed prior to genome integration. The

design of 2LTR-zinc-fingers fused to a GFP moiety (2LTRZFP-GFP) could reduce lentiviral integration by approximately 50% while causing a 100-fold reduction of HIV replication *in vitro* [385], suggesting that simple binding of 2LTRZFP-GFP to pre-integration complex could interfere with HIV integration.

Overall, genome engineering has led to the development of a wide range of promising cutting-edge tools to target HIV-1 infection, and therefore should be considered a valid alternative for patient treatment. The continuous development of these technologies targeted to critical points of HIV infection will lead to design of more improved and safe therapeutic approaches against HIV.

CHAPTER II

Conjugation of TALE activators and suicide lentivectors for targeted elimination of latent HIV cells

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2.1. ABSTRACT

The presence of replication-competent HIV-1 – which resides mainly in resting $CD4^+$ T cells – is a major hurdle to its eradication. While pharmacological approaches have been useful for inducing the expression of this latent population of virus, they have been unable to purge HIV-1 from all its reservoirs. Additionally, many of these strategies have been associated with adverse effects, underscoring the need for alternative approaches capable of reactivating viral expression. Here we show that engineered transcriptional activators based on customizable transcription activator-like effector (TALE) proteins can induce gene expression from the HIV-1 long terminal repeat promoter, and that combinations of TALE activators can synergistically reactivate latent viral expression in cell line models of HIV-1 latency. We further show that complementing TALE activators with class-selective histone deacetylase inhibitors enhances HIV-1 expression in latency models. In addition, we engineered an HIV-responsive suicide lentivector that selectively eliminates HIV latent cells when conjugated with TALE activators. Stimulation of HIV latent cells through TALE activators was required to induce gene expression from suicidal lentivector, leading to cell death of HIV latent cells with minimal hazard to non-infected cells. Collectively, these findings demonstrate that TALE activators are a potentially effective alternative to current pharmacological routes for reactivating latent virus and that combining synthetic transcriptional activators with histone deacetylase inhibitors or suicide lentivectors could lead to the development of improved therapies for latent HIV-1 infection.

Keywords: HIV latency / TALE / synthetic transcription activators / histone deacetylase inhibitors / suicide lentivectors

2.2. INTRODUCTION

Over the past two decades, numerous advances in the treatment of HIV/AIDS have significantly increased the lifespan – and quality of life – of individuals infected with HIV type 1 (HIV-1). Highly active antiretroviral therapy (HAART), in particular, has emerged as a powerful treatment option, capable of decreasing plasma viral loads to below the limit of detection of many clinical assays [30–32]. Yet despite its effectiveness, HAART does not cure patients of HIV-1 infection, due to the existence of residual latent and replication-competent virus hidden in cellular reservoirs [37,49,386–388]. This population of cells, which consists mainly in resting memory $CD4^+$ T cells, harbors integrated proviral DNA that re-emerges shortly after discontinuation of HAART. HIV-1 latency is typically established when activated $CD4^+$ T cells become infected with the virus and revert back to a resting memory state [37]. These cells are thus non-permissive for viral gene expression and refractory to many treatments, including HAART. Although the mechanisms behind latency are complex [37,41], they likely involve: (*i*) the absence of key host transcription factors that drive transcriptional initiation [65,66] or elongation [67,68] in resting $CD4^+$ T cells; (*ii*) low levels of the trans-activator of transcription (Tat) regulatory protein [389]; (*iii*) proviral integration into condensed chromatin regions [54,55] or expressed regions that become silenced by promoter occlusion or collision [57–60]; and (*iv*) the induction of epigenetic modifications that can inhibit viral gene expression, including DNA methylation [62,63] and histone deacetylation [64].

Because the presence of latent HIV-1 represents an enormous barrier toward its eradication, numerous strategies have been developed to purge it from its cellular reservoirs. Chief among these has been activation of latently infected T cells via treatment with cytokines [77] or monoclonal antibodies [74], as well as NF-κB stimulation via protein kinase C agonists [79,80]. Histone deacetylase (HDAC) inhibitors, such as valproic acid [82] and Vorinostat [83], have also proven capable of inducing viral gene expression by disrupting recruitment of HDAC proteins to the HIV long terminal repeat (LTR) promoter [87,88]. These approaches, however, have been unable to eradicate virus from all latent pools and have even been associated with adverse effects, including severe immune reactions [29,52,78,390]. Additionally, the host immune response mediated by CD8+ cytotoxic T lymphocytes (CTL) lacks robustness to clear the HIV infected cells, requiring supplementary stimulation of anti-HIV immune response [91]. As a result, new strategies capable of inducing viral gene expression and enforce infected cell elimination are needed to enable the development of next-generation HIV-1 therapeutics.

The emergence of customizable DNA-binding platforms, including engineered zinc-finger [243] and transcription activator-like effector (TALE) [391] proteins, as well as CRISPR-Cas9 [251], has provided investigators with a set of tools capable of sequence-specific DNA recognition [240]. TALE proteins, in particular, have now been utilized to create a broad range of tools capable of gene modification and regulation, including transcriptional activators [268,269] and repressors [270], nucleases [268,271,272], site-specific recombinases [273] and epigenetic effectors [274–276]. The DNA binding domain of a TALE protein consists of a series of repeat domains, each ~34 amino acid residues in length, that coordinate to recognize a single base pair (bp) via two adjacent amino acid residues, termed repeat variable diresidues (RVDs) [244,245]. A variety of approaches have now been developed that enable rapid construction of custom TALE arrays capable of recognizing nearly any contiguous sequence [265,392]. As a result, TALEs have achieved widespread use throughout biotechnology, with the potential to impact future developments in human gene therapy.

Numerous studies have also demonstrated the utility of genome engineering for combating HIV/AIDs. Specifically, zinc-finger based transcriptional repressors [343–346], in addition to RNA interference [192,393,394], have proven effective at inhibiting HIV replication. Targeted nucleases have also demonstrated the capacity to excise integrated proviral DNA from infected cells [372–374] and confer HIV resistance to cells by inducing knockout of the primary co-receptors for HIV infection [272,289,358,395]. Targeted gene regulation technologies may also prove effective at reversing HIV-1 latency. Specifically, due to their versatility and ability to stimulate robust levels of gene expression in a highly specific manner [396], TALE activators [268] could be used to stimulate viral gene expression within latent HIV-1 reservoirs, providing new means for enabling “shock and kill” therapy. Here we demonstrate that TALE activators can be engineered to recognize the HIV-1 LTR promoter and induce viral gene expression in cell line models of HIV-1 latency. We show that complementing TALE activators with HDAC inhibitors can further enhance TALE-induced activation of latent HIV-1 expression. We also provide evidence that engineered suicide lentivectors can be associated with TALE activators to enforce clearance of stimulated HIV latent cells. These findings indicate that TALE activators and suicide lentivectors are potentially effective tools for reactivating and eliminating latent virus and could contribute to the development of next-generation HIV-1 therapies.

2.3. MATERIALS AND METHODS

2.3.1. TALE activator constructs

Design of HIV-targeted TALEs was performed at TAL Effector-Nucleotide Targeter (TALE-NT) 2.0 web server (https://boglab.plp.iastate.edu/) [397]**.** TALEs were generated as previously described [392,398] using the Golden Gate TALEN and TAL Effector Kit 2.0 (Addgene ID: 1000000024) [265]. Briefly, single RVDs modules were digested with BsaI and ligated in predetermined arrays into pFUS-A and pFUS-B to respectively generate pTLT-A (containing RVD positions 1-10) and pTLT-B (containing RVD positions 11-14). Next, array plasmids pTLT-A and pTLT-B and pLR (last repeat RVD) modules were assembled through digestion with BsmBI and ligation into pcDNANT-T-VP64 [267] to generate pTLT-1 through 10. Correct construction of each plasmid was verified by sequence analysis (**Table S2.1**).

2.3.2. Luciferase reporter constructs

The pTALE activator reporter vectors were constructed through PCR by amplifying the luciferase gene from pGL3-Basic (Promega, Madison, WI, USA) using the primers 5' TALE-Luc-TLT1 through 10, which contained four direct repeats of each TALE binding site and 3' Luc-Rev. PCR products were digested and cloned into the XhoI and SphI restriction sites of pGL3-Basic to generate pGL3-TLT-1 through 10. The HIV-1 LTR reporter plasmid was constructed by PCR amplifying the U3-R region of the 5'LTR promoter from pNL4-3 (NIH AIDS Reagents) [399] using the primers 5' LTR-Fwd and 3' LTR-Rev. PCR product was digested and cloned into the MluI and NheI restriction sites of pGL3-Basic to generate pGL3-LTR. Primer sequences are provided in **Table S2.2**.

The mLTR reporter constructs were generated through PCR mutagenesis of U3 region of the 5'LTR promoter from pNL-GFP-RRE(SA) [188]: wtLTR was generated by PCR using the primers 5' U3-Fwd and 3' R-Rev; mLTR1 was generated by PCR using primers 5' U3-Fwd and 3' -455 U3-Rev; mLTR2 was generated by PCR using primers 5' U3-Fwd and 3' -147/-115 U3-Rev; mLTR3 was generated by PCR using primers 5' U3-Fwd and 3' -183/-115 U3-Rev; mLTR4 was generated by PCR using primers 5' U3-Fwd and 3' -183 U3-Rev; mLTR5 was generated by PCR using primers 5' U3-Fwd and 3' -183/-115 TAR U3-Rev. mLTR1-mLTR5 fragments were assembled by overlap PCR with -115 U3-R region amplified by PCR using primers 5' -115 U3-Fwd and 3' R-Rev. PCR products were digested and cloned into the MluI and NheI restriction sites of pGL3-Basic to generate pGL3-wtLTR through mLTR5.

Correct construction of each plasmid was verified by sequence analysis (**Table S2.3**). Primer sequences are provided in **Table S2.4**.

2.3.3. Suicidal lentivector constructs

HIV suicidal lentivector constructs were generated from the Rev-dependent pNL-GFP-RRE(SA) vector [188] (kindly provided by Dr. Jon W. Marsh, NIMH). pNLR-Fluc was generated by PCR amplifying the firefly luciferase gene from pGL3-Basic using the primers 5' Fluc-Fwd and 3' Fluc-Rev. PCR fragment was digested and cloned into SalI and XbaI restriction sites of pNL-GFP-RRE(SA) (GenBank accession no. EF408805.1). pmNLR-Fluc was generated by amplifying the U3-R regions of mLTR3 promoter from pGL3-mLTR3 through PCR using the primers 5' U3-Fwd and 3' R-Rev. U3-R region was assembled by overlap PCR with U5 region amplified from pNL-GFP-RRE using primers 5' U5-Fwd and 3' U5-Rev. PCR product was digested and cloned into StuI and SalI restriction sites of pNLR-Fluc. pNLR-E2C and pmNLR-E2C were generated by PCR amplifying the E2Crimson [400] gene from pCMV-E2Crimson (Clontech, Mountain View, CA, USA) using primers 5' E2C-Fwd and 3' E2C-Rev, and cloning into SalI and XbaI restriction sites of pNLR-Fluc and pmNLR-Fluc, respectively. pmNLRW-E2C was generated by isolating the WPRE region from FugW (Addgene plasmid #14883) through PCR using the primers 5' WPRE-Fwd and 3' WPRE-Rev and cloning into XbaI restriction site of pmNLR-E2C using the In-Fusion cloning kit (Clontech, Mountain View, CA, USA). pmNLRW-DTA (Diptheria toxin A chain), pmNLRW-LFN (Anthrax Lethal Factor N-terminal), pmNLRW-RTA (Ricin toxin A chain) and pmNLRW-SLO (Streptolysin O) were constructed by cloning the PCR-amplified toxins into SalI and XbaI restriction sites of pmNLRW-E2C. Diptheria toxin A chain (GenBank accession no. AB602359.1) was amplified from FUW-DTA [401] using primers 5' DTA-Fwd and 3' DTA-Rev. Anthrax Lethal Factor N-terminal (GenBank accession no.M29081.1) was amplified from pet-15b LFN-DTA (Addgene plasmid #11075) using primers 5' LFN-Fwd and 3' LFN-Rev. Ricin toxin A chain (GenBank accession no. AASG02053821.1) was amplified from pCAGGS-RTA (kindly provided by Dr. Wendie Cohick, The State University of New Jersey), using primers 5' RTA-Fwd and 3' RTA-Rev. Streptolysin O (GenBank accession no. AB050250.1) was amplified from pBAD-SLO (kindly provided by Dr. Michael Caparon, Washington University) using primers 5' SLO-Fwd and 3' SLO-Rev. Primer sequences are provided in **Table S2.5**.

2.3.4. Cell culture

Human embryonic kidney 293T (HEK293T) (American Type Culture Collection; ATCC) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 2 mM L-glutamine and 1% (v/v) antibiotic-antimycotic (Anti-Anti; Gibco, Carlsbad, CA, USA). Jurkat E6-1 and J-Lat clones (NIH AIDS Reagents) were cultured in RPMI-1640 medium (Life Technologies, Carlsbad, CA) supplemented with 10% (v/v) FBS, 2 mM L-glutamine and 1% (v/v) Anti-Anti. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy anonymous donors through the Scripps Research Institute Normal Blood Donor Program as described [263]. Briefly, PBMCs were isolated using density gradient centrifugation and Ficoll-Paque PLUS (GE Healthcare) according to the manufacturer instructions. Resting $CD4^+$ T cells were purified from PBMCs by negative selection using EasySep custom kit for Human Resting $CD4^+$ T Cell Enrichment Cocktail (Stem Cell Technologies). Unwanted cells were removed using anti-CD8, CD14, CD16, CD19, CD20, CD36, CD123, TCT γ/δ, GlyA, CD66b, CD25, HLA-DR and CD69 magnetic-coated beads. Resting $CD4^+$ T cells were cultured in RPMI-1640 medium supplemented with 10 % (v/v) FBS, 2 mM L-Glutamine and 1% (v/v) Anti-Anti and maintained at 37°C in a humidified atmosphere of 5% CO_2.

2.3.5. Luciferase assays

Luciferase assays were performed as previously described [398]. Briefly, HEK293T cells were seeded onto 96-well plates at a density of 4 x 10^4 cells per well. At 16-24 h after seeding, cells were transfected with 200 ng of plasmid expression construct, 5 ng of pGL3-luciferase reporter construct and 1 ng of pRL-CMV (Promega, Madison, WI, USA) using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. At 48 h after transfection, cells were washed once with Dulbecco's PBS (DPBS; Life Technologies, Carlsbad, CA, USA) and lysed with Passive Lysis Buffer (Promega, Madison, WI, USA). Luciferase expression was measured with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) using a Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA, USA) according to the manufacturer's instructions. Normalized luciferase activity was determined by dividing firefly luciferase activity by Renilla luciferase activity.

2.3.6. TALE activator expression

HEK293T cells were seeded onto a 6-well plate at a density of 5 x 10^4 cells per well. At 16-24 h after seeding, cells were transfected with 5 µg of pTLT-1 through pTLT-10 or pcDNA backbone vector by the calcium phosphate method [402]. At 48 h after transfection, cells were harvested and lysed with RIPA buffer (25 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1 % SDS, pH 7.6) supplemented with EDTA-free Protease Inhibitor Cocktail Tablets (Roche, Basel, Switzerland). The Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA) was used to determine protein concentration according to the manufacturer's instructions. TALE transcription factor expression was analyzed by 4-12% SDS-PAGE (National Diagnostics, Atlanta, GA, USA). Samples were transferred onto a 0.2 µm nitrocellulose membrane as described [403] and detected with Immobilon Western Chemiluminescent HRP substrate (Millipore, Billerica, MA, USA) and Amersham Hyperfilm ECL (GE Healthcare, Little Chalfont, UK) chemiluminescence film. TALE activators were detected by horseradish peroxidase-conjugated anti-HA monoclonal antibody (Clone 3F10; Roche, Basel, Switzerland). β-actin was used as an internal control and detected using a mouse anti-β-actin monoclonal antibody (Clone AC-74; Sigma, St. Louis, MO, USA) and horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Bio-Rad, Hercules, CA, USA) (kindly provided by Dr. Cecília Rodrigues, Universidade de Lisboa).

2.3.7. HIV-1 reactivation

J -Lat cells were seeded onto a 10-cm dish at a density of 1 x 10^5 cells per mL. At 48 h after seeding, 2 x 10^5 cells per transfection were centrifuged at 100 *g* for 10 min at room temperature and resuspended in Nucleofector Solution SE (Lonza, Basel, Switzerland) with 2 µg of pTLT-1 through pTLT-10 or pTat. Cells were transferred to 16-well Nucleocuvette Strips (Lonza, Basel, Switzerland) and electroporated by a 4D-Nucleofector System (Lonza, Basel, Switzerland) using the program CL-120, according to the manufacturer's instructions. J-Lat cells were either left untreated or incubated with 10 ng/µL of TNF-α (R&D Systems, Minneapolis, MN, Canada). At 48 h after transfection, cells were washed twice with DPBS (Life Technologies, Carlsbad, CA, USA) and GFP expression was evaluated by flow cytometry (BD LSR II Flow Cytometer System; BD Biosciences, Franklin Lakes, NJ, USA). For each sample, 10,000 live events were collected, and data was analyzed using FlowJo (Tree Star, Inc., San Carlos, CA, USA).

2.3.8. HDAC inhibitor treatments

HDAC inhibitors were kindly provided by Dr. Richard Barnard and Dr. Daria Hazuda (Merck Research Laboratories, Pennsylvania, USA) J-Lat cells were seeded onto a 10-cm dish at a density of 2 x 10^5 cells per mL. At 48 h after seeding, 1 x 10^6 cells per transfection were centrifuged at 100 x g for 10 min at room temperature and resuspended in Nucleofector Solution V (Lonza, Basel, Switzerland) with 4 µg of pTLT-5 through pTLT-8. Cells were transferred to a Nucleocuvette (Lonza, Basel, Switzerland) and electroporated with an Amaxa Nucleofector II Device (Lonza, Basel, Switzerland) using the program X-001 according to the manufacturer's instructions. At 24 h after transfection, J-Lat cells were treated with DMSO 0.1%, SAHA (330 nM, 660 nM or 1 µM), MRK-1 (660 nM, 1 µM or 2 µM), MRK-11 (3 µM, 5 µM, 20 µM) [84] or H-12 (1 µM, 3 µM or 5 µM) for 24 h. After treatment, cells were washed twice with DPBS and GFP expression was evaluated by flow cytometry analysis (BD LSR II Flow Cytometer System; BD Biosciences, Franklin Lakes, NJ, USA). For each sample, 10,000 live events were collected, and data was analyzed using FlowJo (Tree Star, Inc., San Carlos, CA, USA). The selective IC50 activity for each HDAC inhibitor is presented in **Table S2.5**.

2.3.9. Lentivirus production

Pseudo-typed HIV NL4-3Δenv-EGFP and second-generation NLR-E2C reporter lentivectors were generated HEK293T cells using the Lipofectamine 3000 optimized protocol for lentiviral production (Life Technologies, Carlsbad, CA, USA). HEK293T cells were seeded in 6-well plates at a density of 1.2 x 10^6 cells per well for 24 hours. VSVg-pseudotyped NL4-3ΔenvEGFP was produced by transfection with 3.3 µg of pNL4-3ΔenvEGFP (NIH AIDS Reagents) [404] and 0.3 µg of VSVg envelope pMD2.G. VSVg-pseudotyped mNLR-E2C and mNLRW-E2C lentivirus were produced by transfection with 1.8 µg of pmNLR-E2C or pmNLRW-E2C constructs respectively, 1.5 µg of packaging plasmid psPAX2 (Addgene plasmid # 12260; Funcional lentivirus - LV) or psPAX2-D64V (Addgene plasmid # 63586; Integrase-deficient lentivirus - IDLV) and 0.3 µg of VSVg envelope pMD2.G (Addgene plasmid # 12259). Cell medium was replaced 6 hours after transfection. Lentivirus present in the cell supernatant were collected after 24 hours and cleared form cell debris by centrifugation at 500 *g* for 10 min at room temperature and stored at -80 ºC. Viral production was quantified by a p24 capture ELISA assay (NCI-Frederick Cancer Research and Development Center – AIDS Vaccine Program kit, NIH, USA), according to manufacturer's instructions.

2.3.10. HIV/Suicide lentivector co-transduction

Jurkat cells were seeded onto 24-well plates with 5 x 10^5 cells per well. Cells were transduced with VSVg-pseudotyped NL4-3Δenv-EGFP at an MOI of 2 (20 ng p24) through spinoculation by centrifugation (800 *g*, 60 min, 32 ºC) in the presence of polybrene (8 µg/mL). Plates were incubated at 37 ºC for 4 hours and cell medium was replaced. Twenty-four hours after infection, 1 x 10^5 of non-infected or infected cells were transduced with VSVg-pseudotyped mNLR-E2C lentivirus (20 ng p24), mNLRW-E2C lentivirus (20 ng p24) or integration-deficient lentivirus (50 ng p24) through spinoculation (800 *g*, 60 min, 32 ºC) in the presence of polybrene (8 µg/mL). Plates were incubated at 37 ºC for 4 hours and cell medium was replaced. At 48 hours after transduction, cells were washed twice with DPBS (Life Technologies, Carlsbad, CA, USA). E2-Crimson and GFP expression was evaluated by flow cytometry (BD Accuri C6 Flow Cytometer System; BD Biosciences, Franklin Lakes, NJ, USA). For each sample, 10,000 live events were collected, and data was analyzed using FlowJo (Tree Star, Inc., San Carlos, CA, USA).

2.3.11. TALE/Suicide lentivector-mediated cell death analysis

Jurkat or J-Lat 10.6 cells were seeded onto a 10-cm dish at a density of 1 x 10^5 cells per mL. At 48 h after seeding, 2 x 10^5 cells per transfection were centrifuged at 100 *g* for 10 min at room temperature and resuspended in Nucleofector Solution SE (Lonza, Basel, Switzerland) with 1 µg of pTLT5-7 and 1 µg of suicide lentivector. Cells were transferred to 16-well Nucleocuvette Strips (Lonza, Basel, Switzerland) and electroporated by a 4D-Nucleofector System (Lonza, Basel, Switzerland) using the program CL-120, according to the manufacturer's instructions. At 48 h after transfection, cells were washed twice with DPBS (Life Technologies, Carlsbad, CA, USA) and apoptotic cells were stained with Annexin V (Life Technologies, Carlsbad, CA, USA), according to manufacturer instructions. Annexin V staining was evaluated by flow cytometry (BD LSR II Flow Cytometer System; BD Biosciences, Franklin Lakes, NJ, USA). For each sample, 10,000 events were collected, and data was analyzed using FlowJo (Tree Star, Inc., San Carlos, CA, USA).

2.3.12. Statistical analysis

Statistical analyses for all experiments were performed from three independent experimental replicates ($n = 3$) unless otherwise indicated. Two-tailed Student's *t*-test was used for paired and unpaired samples (Prism Software 5.0, GraphPad Software).

2.4. RESULTS

2.4.1. Designing TALE activators to target the HIV-1 promoter

We sought to reverse HIV-1 latency by inducing viral gene expression using engineered TALE activators. We constructed ten TALE proteins designed to recognize distinct 16-bp sites within the HIV-1 LTR, the region of the virus that serves as its promoter **(Fig. 2.1A)**. TALE binding sites were constrained only by the presence of a 5' thymidine (T_0) nucleotide [267]. We fused each synthetic TALE array to VP64 [254], a tetrameric repeat of the herpes simplex virus VP16 transactivation domain, to generate synthetic transcriptional activators. VP64 is a widely used transactivation domain [241] capable of recruiting host cellular transcription factors to targeted genomic loci [405,406], but does not activate gene expression alone [254]. Each TALE transcription factor contained a C-terminal hemagglutinin (HA) tag and an internal nuclear localization signal (NLS) sequence between the DNA binding and transactivation domains **(Fig. 2.1A)**. The amino acid sequence of each protein is presented in **Table S2.1**.

In order to determine whether each TALE could recognize its intended target site and induce gene expression, we adapted a previously described transient reporter assay [398] that correlates TALE-induced gene activation with increased luciferase expression. We inserted four direct repeats of each LTR binding site upstream of a luciferase reporter gene and co-transfected human embryonic kidney (HEK) 293T cells with reporter plasmid and expression vectors for each TALE activator **(Fig. 2.1C and 2.1D)**. This strategy was undertaken in order to increase reporter gene expression and more accurately evaluate TALE activity. Eight of the ten TALE activators (all but TLT4 and TLT8) induced a >800-fold increase in luciferase expression, with TLT1 (~3,400-fold), TLT3 (~2,900-fold), TLT7 (~2,500-fold) and TLT9 (~2,200-fold) inducing the highest levels of activation ($p < 0.001$) **(Fig. 2.1D)**. TLT4 and TLT8 achieved similarly high levels of absolute luciferase activity, but induced a modest ~100-fold increase in activation over mock-transfected cells. Even in the absence of a TALE activator, transfection of the TLT4 and TLT8 reporter plasmids led to a significant increase in luciferase expression ($p < 0.001$) (data not shown). Not surprisingly, however, the binding sites for TLT4 and TLT8 overlap with those recognized by the endogenous transcription factors C/EBP and NF-κB [407] **(Fig. 2.1A)**, respectively, indicating that native proteins could have been contributing to reporter gene activation. Compared to the reporter plasmid alone, increasing luciferase expression was evident after co-transfection with the specific TALE activator, indicating that TALEs have the potential to outcompete endogenous transcription factors for LTR binding sites.

Western blot analysis of HEK293T lysates also revealed that each TALE activator was well expressed **(Fig. 2.1D)**. Low levels of a non-specific band (~70 KDa), however, were detected in several samples, possibly due to translation of a second open-reading frame present within the TALE mRNA transcript or recombination within the TALE DNA-binding domain, a phenomenon that can occur within a highly repetitive motif [408].

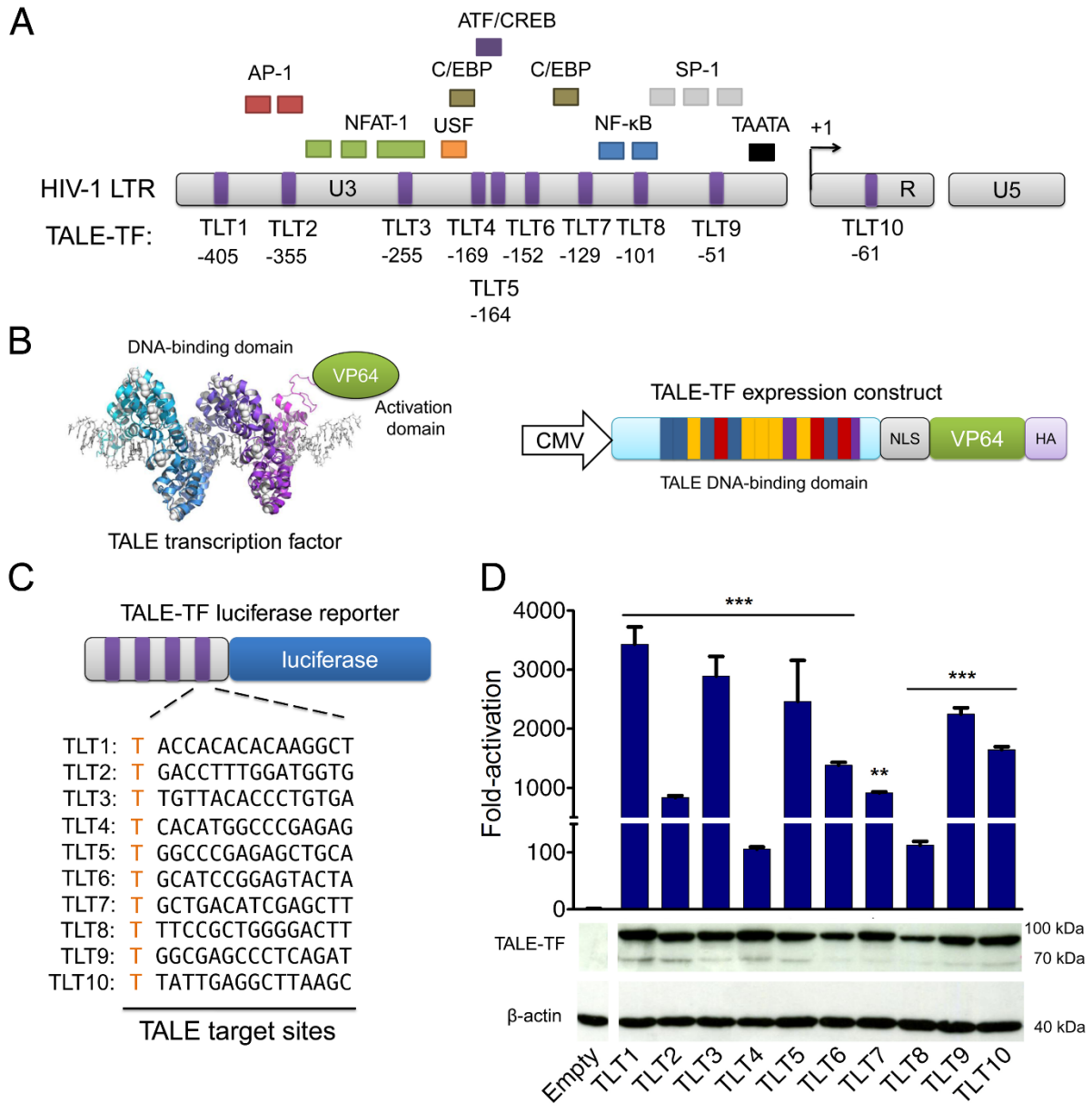

Figure 2.1- TALE activators designed to target the HIV-1 LTR promoter. (A) Schematic representation of the TALE transcription activator (TLT) binding sites within the HIV-1 long terminal repeat (LTR) promoter relative to the transcriptional start site (TSS) and main endogenous transcription factor binding sites. **(B) (Left)** Cartoon illustrating the structure of a TALE activator, adapted from [285]. TALE repeats are colored cyan and purple, DNA shown as grey sticks. **(Right)** Schematic representation of the TLT expression construct used in this study. CMV indicates the cytomegalovirus promoter, TALE repeats are shown as individual bars (14.5 repeats total), VP64 denotes the tetrameric repeat of the herpes simplex virus VP16 transactivation domain, NLS stands

for the nuclear localization signal derived from the simian virus (SV40) and HA indicates the hemagglutinin A tag. **(C)** Schematic representation of the luciferase reporter system containing four direct repeats of the TALE target sites for each TALE activator. Each TALE target site is shown. **(D) (Top)** Fold-activation of luciferase expression after co-transfection of TALE activators with luciferase reporter plasmid into HEK293T cells. Luciferase expression was normalized to cells transfected with reporter plasmid only. *Renilla* luciferase expression was used to normalize for transfection efficiency and cell number. Error bars indicate standard deviation of one experiment with three transfection replicates ($n = 3$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; *t*-test sample vs control (4x TALE binding site vector only; Empty)). (**Bottom)** Western blot of lysate from HEK293T cells transfected with TALE activators. Samples were taken 48 h after transfection and probed with horseradish peroxidase-conjugated anti-HA and anti-β-actin (loading control) antibodies. Empty indicates lysate from HEK293T cells transfected with empty pcDNA vector only.

2.4.2. TALE activators activate gene expression from the HIV LTR

We next set out to test the ability of each TALE activator to stimulate transcription from the full-length U3 and R regions of the HIV-1 LTR using an episomal reporter assay. The U3-R regions of the LTR contain the core promoter, enhancer and modulatory region, and regulate viral expression. Notably, unlike the transient reporter assay described above, which asked whether each TALE protein could bind its intended DNA target, this analysis aimed to evaluate the ability of each TALE activator to stimulate transcription from the full-length HIV-1 promoter.

HEK293T cells were co-transfected with TALE activator and a reporter vector that contained the sequence between -455 and +96 from the LTR transcriptional start site (TSS) upstream of a luciferase reporter gene **(Fig. 2.2)**. We co-transfected separately an expression vector encoding the HIV-1 Tat protein and the reporter vector as positive control. Multiple activators, including TLT4, 5, 6, 7 and 8, induced a 7.5- to 14-fold increase in luciferase activity ($p < 0.01$), while Tat yielded only a ~7-fold increase in activation **(Fig. 2.2)**, likely because it stimulates transcriptional elongation more efficiently than initiation [409].

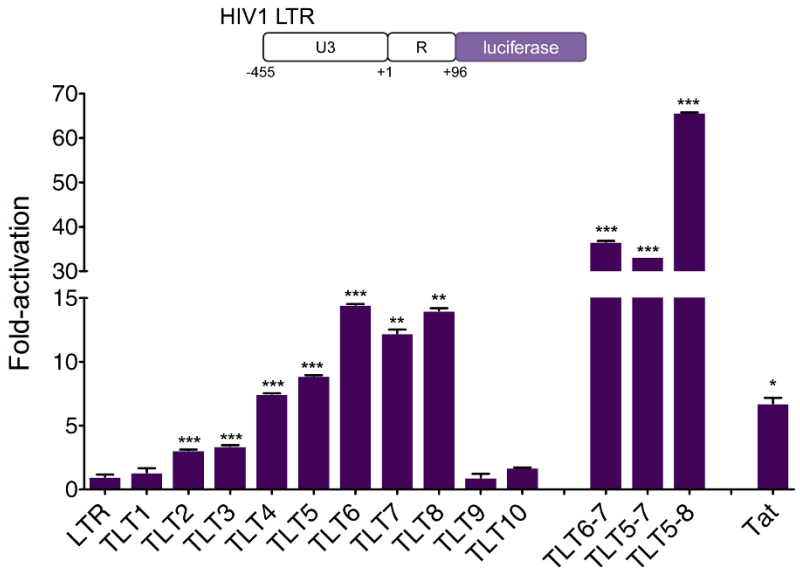

Figure 2.2- TALE-mediated gene activation from the HIV-1 LTR promoter. (Top) Schematic representation of the luciferase reporter system used to evaluate TALE activator activity from the HIV-1 LTR promoter. The U3 and R regions of the HIV LTR were placed upstream of the luciferase reporter. **(Bottom)** Fold-activation of luciferase expression in HEK293T cells co-transfected with reporter plasmid and TALE activator or Tat expression vectors. Luciferase expression was normalized to cells transfected with reporter plasmid only. Error bars indicate standard deviation of one experiment with three transfection replicates ($n = 3$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; *t*-test sample vs. control (LTR)).

Previous reports have demonstrated that co-delivery of combinations of TALE transcription factors can lead to a synergistic increase in gene expression via cooperative effects that could mimic those associated with natural transcriptional processes [302,305]. The most potent TALE activators, TLT4, 5, 6, 7 and 8, were designed to recognize a small region of the LTR between -170 and -100 bp from the TSS **(Fig. 2.1A)**. We thus co-transfected HEK293T cells with LTR reporter plasmid and different combinations of TALE activators to test whether these proteins could be used in tandem to further enhance gene expression. Increased gene activation was observed for each set tested, most notably with a ~70-fold increase in luciferase expression after co-transfection with TLT5, 6, 7 and 8 (hereafter referred to as TLT5-8) ($p < 0.001$) **(Fig. 2.2)**. Overall, these data demonstrate that TALE activators designed to target the U3 and R regions of the HIV LTR promoter can induce efficient gene activation.

2.4.3. Reactivation of latent HIV-1 by TALE activators

We next asked whether TALE activators could reactivate viral expression in a cell line model of HIV-1 latency. To explore this, we used the Jurkat-derived J-Lat lymphocytic cell lines, which harbor a full-length integrated HIV-1 proviral genome containing a GFP gene that serves as a reporter for viral gene expression (HIV1-ΔEnv-GFP) **(Fig. 2.3A)**. J-Lat cells poorly express the integrated proviruses under normal conditions, but viral gene expression can be efficiently induced by stimulation using tumor necrosis factor (TNF)-α [55]. Since each J-Lat clone is derived from a unique HIV-1 integration event, they display differential levels of gene

and/or chromatin repression, as demonstrated by their distinct gene activation thresholds after TNF-α stimulation [410].

We nucleofected J-Lat 10.6 cells, which display a low viral gene activation threshold, with expression vectors encoding TALE activators or Tat and evaluated HIV-1 expression by measuring the percentage of GFP-positive cells by flow cytometry **(Fig. 2.3A)**. As expected, cells treated with TNF-α or transfected with Tat showed robust reactivation, with upwards of 55% and 75% of cells producing GFP, respectively. Among all individual TALE activators tested, TLT5 and 6 (~40% GFP-positive cells each) and TLT7 and 8 (~30% GFP-positive cells each) yielded the highest levels of expression, with cells transfected with TLT5-8 also showing upwards of 50% GFP-positive cells ($p < 0.05$) **(Fig. 2.3A)**. Nucleofection of an empty vector (pcDNA) resulted in minor (~5%) reactivation, indicating that stress from the nucleofection process can also contribute to reactivation **(Fig. 2.3A)**. Sequence analysis of different strains of HIV-1 subtype B (i.e. the most predominant subtype across Europe, America, Australia and Japan) revealed that the binding sites for these TALEs are generally well conserved, with increasing preservation from TLT5 to TLT8 **(Table S2.7)**.

The relative potencies of the TALE activators in J-Lat 10.6 cells correlated with their ability to stimulate transcription in the reporter assay used in **Fig. 2.2**. Analysis of mean fluorescence intensity (MFI) in transfected J-Lat 10.6 cells further indicated that each TALE activator induced approximately a 10 to 15-fold increase in viral gene expression (**Fig. S2.1A**). Interestingly, in contrast to the episomal studies presented in **Fig. 2.2**, TLT5-8 yielded a similar number of GFP positive cells as the combinations TLT6-7 and TLT5-7 **(Fig. 2.3A)**. One possible explanation for this is that measuring the number of GFP positive J-Lat 10.6 cells may not necessarily afford the amount of sensitivity needed to distinguish between the potencies of specific combinations of activators. Indeed, analysis of MFI in J-Lat 10.6 cells revealed that TLT5-8 induced higher amounts of viral gene expression (~20-fold compared to the negative control) than TLT7 or the combinations TLT6-7 and TLT5-7 (~14-fold compared to the negative control) (**Fig. S2.1B**).

To test the versatility of the TALE activators, we next evaluated their ability to induce HIV-1 transcription in the J-Lat clones 6.3, 8.4 and 9.2, which each possess a higher gene activation threshold than J-Lat 10.6 cells [55]. Sequence mapping of these clones previously revealed that the HIV-1 provirus is integrated into actively transcribed genes, unfavorable to HIV transcription [58,411]. We observed significant reactivation in each cell line tested after co-transfection with TLT5-8 ($p < 0.05$) but at rates much lower than by TNF-α stimulation **(Fig.**

2.3B), indicating that the level of repression within latently-infected cells can influence the ability of TALEs to mediate activation.

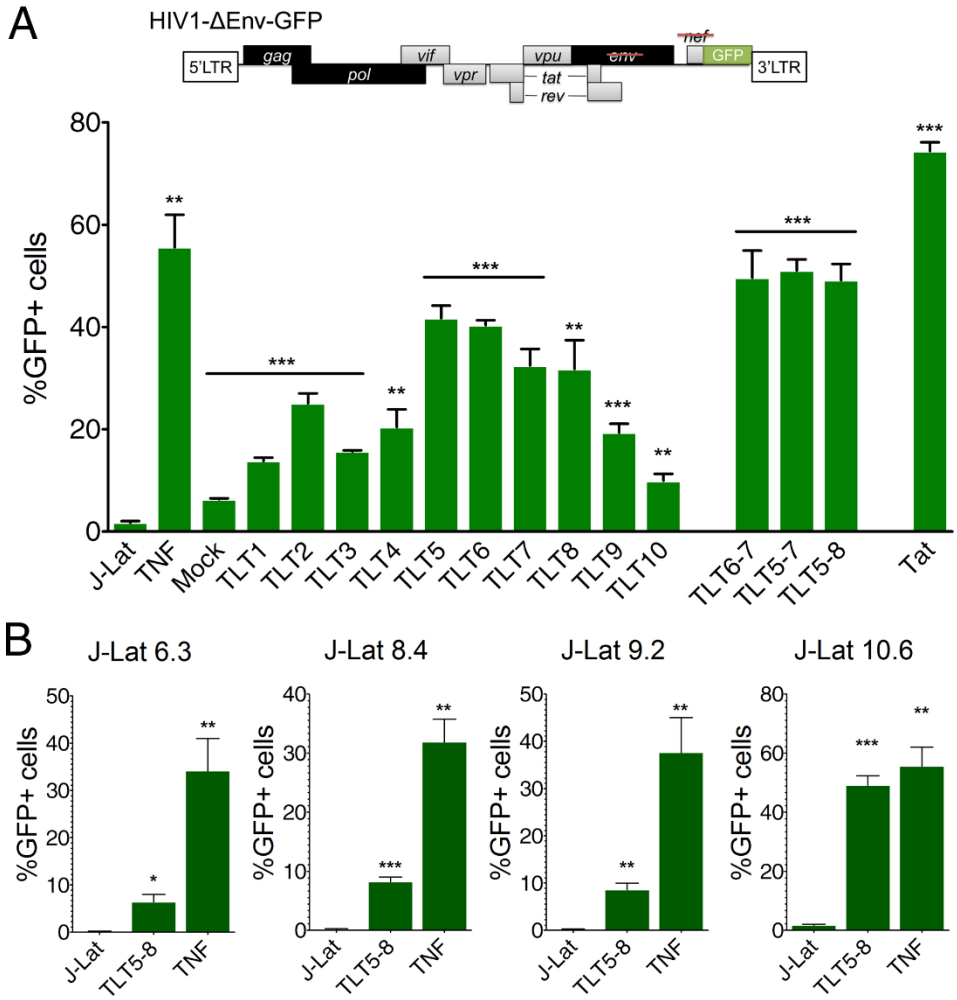

Figure 2.3- Reactivation of latent HIV-1 expression by TALE activators in multiple cell line models of HIV-1 latency. (A) (Top) Schematic representation of the HIV-1 proviral genome present in J-Lat cells. Full-length HIV-1 was derived from the molecular clone pNL4-3-ΔEnv-GFP and expresses a GFP gene from the LTR promoter. Structural viral genes are shown in black, auxiliary genes are shown in grey. The *nef* and *env* genes were inactivated to force a single infection cycle. **(Bottom)** Percentage of GFP positive J-Lat 10.6 cells after nucleofection with TALE-TF and Tat expression plasmids, or treatment with TNF-α (10 ng/μL). GFP positive cells were measured by flow cytometry 48 h after nucleofection. "J-Lat" indicates non-transfected J-Lat 10.6 cells. "Mock" indicates cells transfected with an empty pcDNA backbone. Error bars indicate standard deviation of three independent experiments (n = 3). **(B)** Percentage of GFP positive J-Lat 6.3, 8.4 and 9.2 cells after nucleofection with TLT5-8 and Tat expression plasmids, or treatment with TNF-α (10 ng/μL). GFP positive cells were measured by flow cytometry 48 h after nucleofection. "J-Lat" indicates non-nucleofected cells. Error bars indicate standard deviation (n = 3; *p < 0.05; **p < 0.01; ***p < 0.001; t-test sample vs control (J-Lat)).

2.4.4. Combining TALE activators with HDAC inhibitors enhances latent HIV activation

We next explored the possibility of enhancing HIV-1 reactivation by combining TALE activators with a histone deacetylase (HDAC) inhibitor. Because HIV-1 proviral integration in J-Lat clones favors heterochromatic regions, especially those near alphoid DNA repeat elements [55], we hypothesized that chromatin remodeling by HDAC inhibition could enhance TALE binding to the HIV-1 LTR, thereby increasing viral gene expression. Indeed, previous reports have indicated that the LTR promoter is typically hypoacetylated and that treatment with HDAC inhibitors can lead to the recruitment of the transcriptional machinery to the HIV-1 promoter [64], as well as activation of the positive transcription elongation factor b (P-TEFb), which can stimulate viral transcriptional elongation [412,413]. Moreover, multiple studies have shown that combining HDAC inhibitors with other compounds also capable of reversing HIV latency can synergistically increase viral reactivation across a variety of repression states [414–417].

We transfected J-Lat 6.3 and 10.6 cells, which each display distinct activation thresholds, with TLT5-8 and treated each population with increasing concentrations of HDAC inhibitors selective towards specific enzyme types: Vorinostat (i.e., suberoylanilide hydroxamic acid or SAHA), strongly inhibits class I HDACs (1, 2, 3) but also has modest activity against class II (6, 10, and 11); MRK-1, selectively targets class I (1, 2 and 3) and class II HDAC6; MRK-11, selective for class II HDAC6 HDACs (4, 5, 6 and 7) and class I HDAC8; and H-12, highly selective for class I HDAC1 and HDAC2 only. SAHA, in particular, is an FDA-approved HDAC inhibitor that has been shown to induce viral transcription in latent $CD4^+$ T cells from HIV-infected patients [87,88] (though it was unable to increase HIV-1 production [87]). The HDAC inhibitors IC50 activity is presented in **Table S2.6**. Based on previous studies [418,419], we used a specific range of HDAC inhibitor concentrations that would show minimal activity without inducing significant cell death. Compared to cells transfected with TLT5-8 alone, we observed a significant increase ($p < 0.05$) in HIV-1 expression upon co-treatment with SAHA, MRK-1 and MRK-11 **(Fig. 2.4)**. Specifically, reactivation was evident in up to ~23% and ~75% of J-Lat 6.3 and 10.6 cells, respectively, corresponding to a 3.5- and 2-fold increase in HIV-1 transcription **(Fig. 2.4)**. In contrast, combining TLT5-8 with H-12 inhibitors caused no significant increase in HIV expression in all cell lines, indicating that HDAC-1 and -2 suppression alone is not sufficient to further stimulate latent HIV expression. We also observed increased levels of HIV-1 expression in J-Lat 8.4 and 9.2 cells co-treated with TLT5-8 and

HDAC inhibitors, but these values were neither significant nor dose-dependent **(Fig. S2.2)**, showing that cooperation between TALE activators and HDAC inhibitors was context-dependent. Analysis of MFI in treated J-Lat 10.6 cells also revealed a significant and dose-dependent increase in viral gene expression after co-treatment with TLT5-8 and SAHA **(Fig. S2.3)**. Cells treated with only SAHA, MRK-1 and MRK-11 yielded a negligible increase in reactivation except for MRK-1 in J-Lat 10.6 clone (~30% with 2 µM of MRK-1). Collectively, these results demonstrate that complementing TALE activators with HDAC inhibitors can lead to enhanced reactivation of latent HIV-1 expression.

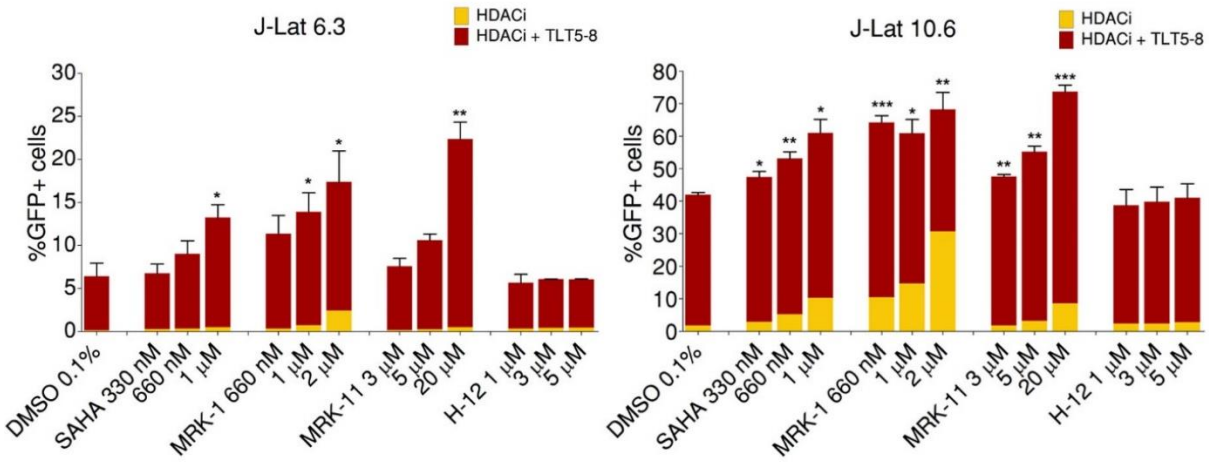

Figure 2.4- Enhanced reactivation of latent HIV-1 expression by combining TALE activators with a histone deacetylase inhibitor. Percentage of GFP-positive J-Lat 6.3 and 10.6 cells after nucleofection with TLT5-8 expression plasmids and treatment with increasing concentrations of SAHA (330 nM, 660 nM, 1 µM), MRK-1 (660 nM, 1 µM, 2 µM), MRK-11 (3 µM, 5 µM, 20 µM), H-12 (1 µM, 3 µM, 5 µM) or DMSO (0.1%) control for 24 h. GFP-positive cells were measured by flow cytometry 48 h after nucleofection. Error bars indicate standard error of the mean of three independent experiments (n = 3; *p < 0.05; **p < 0.01; ***p < 0.001; t-test sample vs control (DMSO)).

2.4.5. TALE activators do not activate primary resting CD4$^+$ T cells

Activation of CD4$^+$ T cells during induction of latent HIV expression has the potential to trigger a generalized immune response [52]. To determine whether TALE activators designed to bind the HIV LTR can inadvertently trigger T cell activation, we nucleofected primary resting CD4$^+$ T cells with TLT5-8 or an empty pcDNA backbone vector and measured the percentage of cells CD25 activation marker. As expected, resting CD4$^+$ T cells stimulated with a cocktail of anti-CD3/CD28 expressed high levels of CD25 (~75%), while less than 2% of cells transfected with TLT5-8 expressed CD25 **(Fig. 2.5A)**. Resting CD4$^+$ T cells treated with 1 µM of SAHA also displayed a negligible increase in CD25 expression **(Fig. 2.5A)**. Forward

and side scatter (FSC/SSC) gating of live cells revealed that $CD4^+$ T cells transfected with TLT5-8 showed no decrease in cell viability compared to mock transfected cells **(Fig. 2.5B)**. Taken together, these results indicate that TALE activators do not dramatically increase the expression of $CD4^+$ T cell activator markers or impact cell viability.

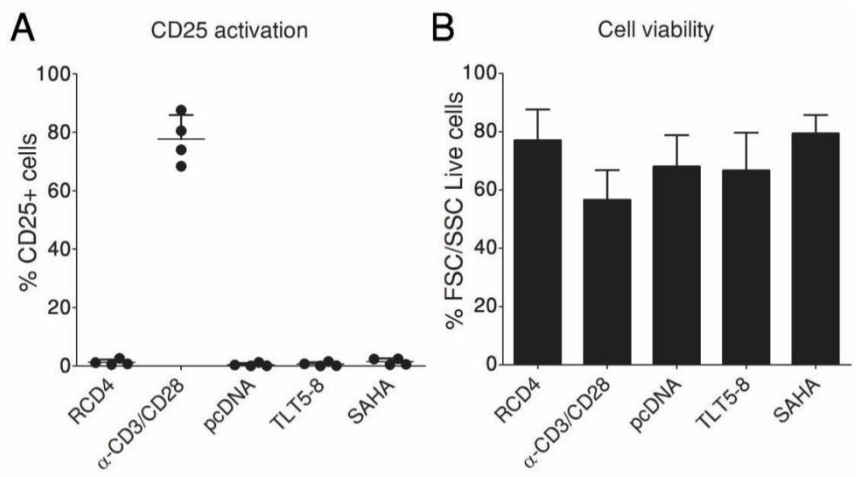

Figure 2.5- Cell-activation mark expression and cell viability analysis of primary resting CD4+T cells in the presence of TALE activators. (A) Percentage of CD25-positive-resting $CD4^+$ T cells after nucleofection with empty pcDNA, and TLT5-8 expression plasmids or treatment with SAHA. **(B)** Percentage of viable resting $CD4^+$ T cells measured by forward and side scatter (FSS/SCC) gating after nucleofection with empty pcDNA and TLT5-8 expression plasmids or treatment with SAHA. RCD4 indicates unstimulated cells; αCD3/CD28 indicates cells stimulated with anti-CD3/CD28 beads. Cells were analyzed by flow cytometry 72 h after nucleofection. Error bars indicate standard error of the mean (*n*= 4 independent donors).

2.4.6. Engineering of suicidal lentivectors to clear latent HIV cells stimulated by TALE activators

Despite the potential of TALE synthetic activators to stimulate latent viral expression, it does not guarantee elimination of HIV reservoirs particularly due to lack of efficient CTL-mediated immune response [91]. For this purpose, we explored a suicidal gene therapy approach to selectively kill latent HIV cells reactivated by our TALE activator technology. We engineered a previously developed HIV-reporter lentivector (pNL4-3-GFP-RRE) for conjugation with our TALE activator technology. This vector incorporates a GFP reporter responsive to the presence of Tat and Rev regulatory proteins typically expressed during HIV replication cycle [188]. Tat and Rev regulatory function in HIV-1 replication is described in section 1.1.2.1. We modified pNL4-3-GFP-RRE to design a suicidal lentivector incorporating a toxin (pmNLR-Toxin; **Fig. 2.6A**) that could be conjugated with HIV-targeted TALE activators previously designed in Chapter II and clear reactivated latent cells through a "shock and kill" gene-based approach **(Fig. 2.6B)**.

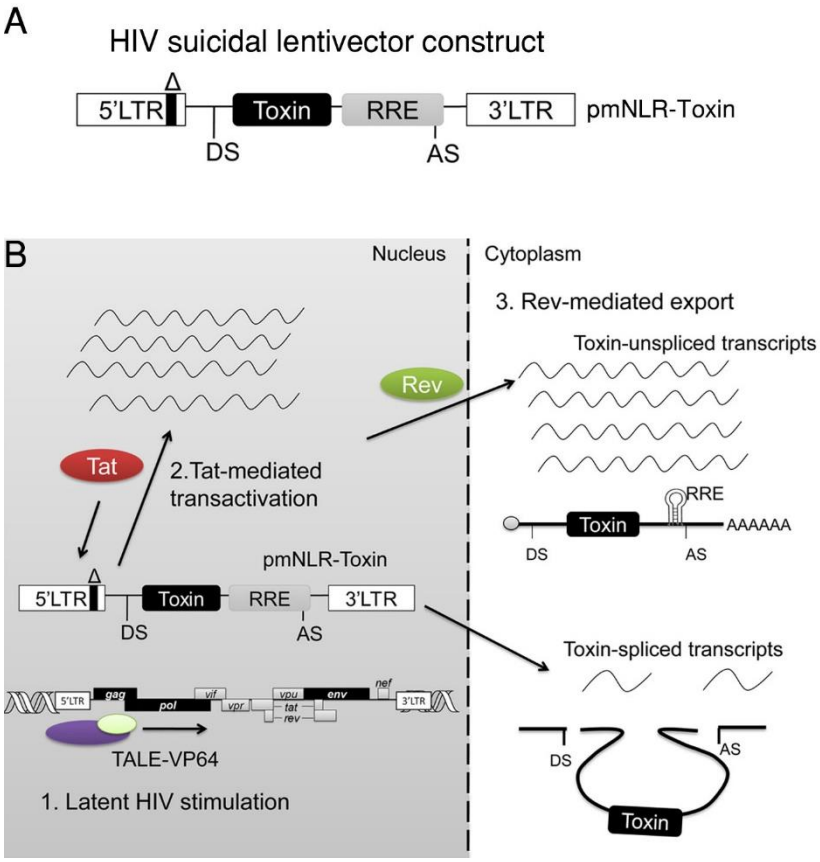

Figure 2.6– "Shock and kill" gene-therapeutic strategy to selectively eliminate HIV-1 latent cells. (A) Schematic illustration of the HIV suicide lentivector construct. Lentiviral HIV-responsive suicidal vector contains a toxin gene under the control of Tat-responsive LTR and a Rev-responsive element (RRE) between donor (DS) and acceptor (AS) splicing sites of HIV-1. The 5'LTR promoter is modified (Δ) to prevent TALE activator recognition and undesirable gene expression in non-infected cells. **(B)** Schematic representation of the strategy proposed for cell-specific elimination of HIV-1 latently infected cells. Cells are co-transfected with TALE activators (TALE-VP64) and a suicidal lentivector (pmNLR-Toxin). TALE activators specifically target the HIV LTR promoter and recruit the transcription machinery to induce latent viral expression (1). HIV-responsive suicidal lentivector harbors a suicide gene (Toxin) conditioned to the presence of HIV Tat and Rev proteins. In the absence of Tat and Rev, any leaking transcripts from the suicidal vector in will be excised due to the presence of HIV-1 splicing sites, eliminating the toxic gene. In HIV-1 infected cells, Tat expression will enhance the suicidal vector transcription from the 5´LTR (2), while Rev expression will promote the export of the unspliced mRNA to the cytoplasm (3), enabling toxin expression and mediated killing of infected cells.

Both 5'LTR from integrated HIV provirus and suicidal lentivectors vector are identical, so it is expected that TALE activators should likewise bind the suicidal vector and lead to undesirable gene activation. In addition, transcriptional background from the 5'LTR promoter posed a major obstacle to HIV suicidal gene therapy due to unspecific cell death of non-infected cells [420–422]. Initial studies characterizing the 5'LTR demonstrated that mutations at the modulatory region from -187 to -112 bp relative to TSS reduced background transcription without affecting Tat activity [423]. On this principle, we designed luciferase reporter constructs under the control of modified 5'LTR promoters (mLTR1-mLTR5) to disrupt binding of TALE activators (TLT5, TLT6 and TLT7) **(Fig. 2.7A)**. This strategy was carried to reduce basal activity and simultaneously prevent TALE-mediated activation without affecting Tat

transactivation. Four of the mLTR constructs (mLTR1, mLTR3, mLTR4 and mLTR5) showed far reduction of TALE-mediated activation (~2-fold) compared to native 5'LTR (wtLTR; ~65-fold). Among these, mLTR3 – for which U3 modulatory region from -183 to -115 bp relative to TSS was substituted for an irrelevant segment – showed a slight decrease of background transcription and increased Tat-mediated activation in comparison to wtLTR **(Fig. 2.7A)**. Consequently, we selected mLTR3 to drive gene expression from our suicidal lentivector construct.

We next evaluated gene expression from the Tat/Rev-dependent suicidal lentivector incorporating a firefly luciferase (Fluc) reporter driven by the native wtLTR (pNLR-Fluc) or modified mLTR3 (pmNLR-Fluc) in HEK293T cells **(Fig. 2.7B; Left)**. Despite the absence of Tat and Rev proteins, TLT5-7 enhanced luciferase activation (~12-fold) in pNLR-Fluc, while in pmNLR-Fluc a slight increase (~4-fold) was observed possibly due to unspecific activation caused by the presence of VP64. On the other hand, mLTR3-driven pmNLR-Fluc activation (~53-fold) in the presence of Tat and Rev was superior to that observed with wtLTR-driven pNLR-FLuc (~23-fold) **(Fig. 2.7B; Right)**. These results indicate that incorporation of mLTR3 turn activation of HIV suicidal lentivector far more specific to the presence of Tat and Rev viral proteins.

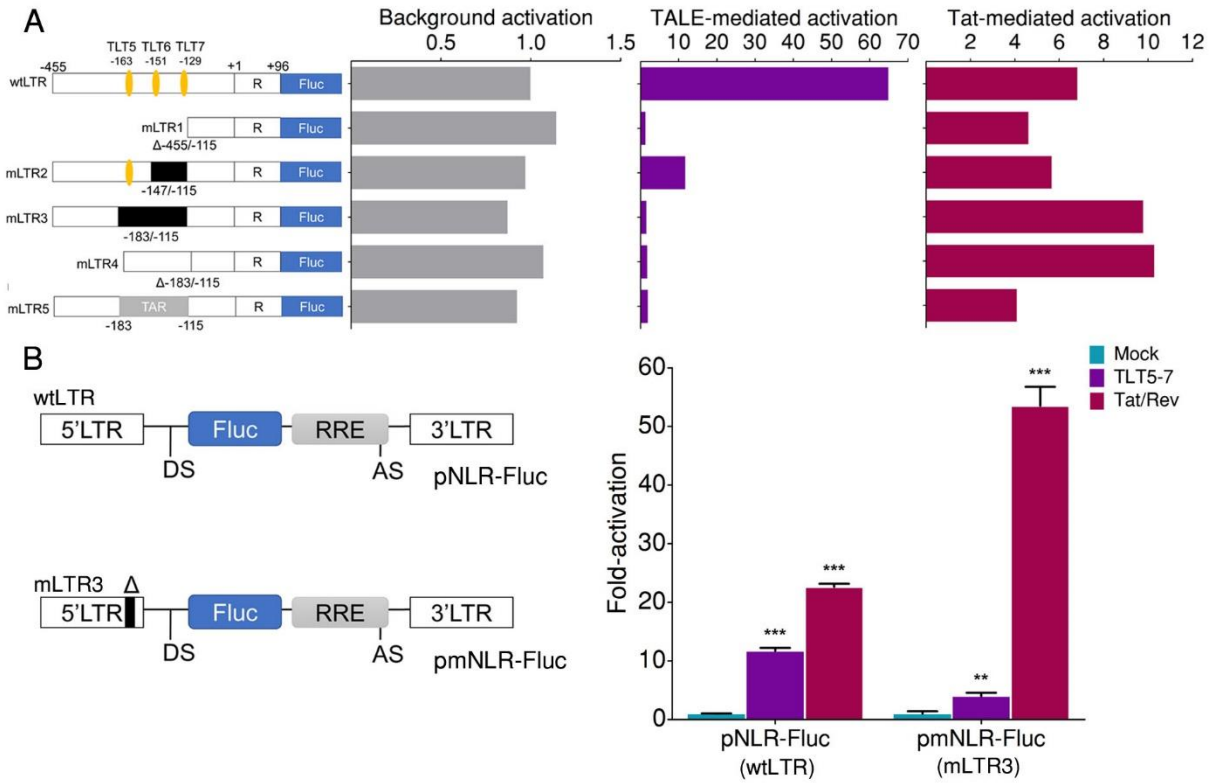

Figure 2.7– Engineering LTR promoter of Tat/Rev-dependent suicide lentivector for conjugation with TALE activators. (A) Design of luciferase reporter constructs driven by LTR mutants (mLTR) to disrupt TALE-mediated activation. TALE activators (TLT5, TLT6 and TLT7) binding sites relative to the transcriptional start site (TSS) are illustrated. The U3 and R regions of the HIV LTR were placed upstream of the luciferase reporter: wtLTR corresponds to the native full-length U3 and R regions; in mLTR1 the U3 segment upstream of -115 bp was entirely deleted; in mLTR2 the U3 segment from -147 to -115 bp was substituted for an irrelevant segment; in mLTR3 the U3 segment from -183 to -115 bp was substituted for an irrelevant segment; in mLTR4 the U3 segment from -183 to -115 bp was entirely deleted; in mLTR5 the U3 segment from -183 to -115 bp was substituted for Tat-binding trans-activating response element (TAR). Fold-activation of luciferase expression was evaluated in HEK293T cells co-transfected with native (wtLTR) or modified mLTR (mLTR1-mLTR5) luciferase constructs and empty pcDNA vector (Background activation), TLT5-7 constructs (TALE-mediated activation) or Tat-expression plasmids (Tat-mediated activation). Luciferase expression was normalized to cells co-transfected with pcDNA control. *Renilla* luciferase expression was used to normalize for transfection efficiency and cell number. **(B) (Left)** Schematic representation of the suicide lentivectors incorporating a firefly luciferase (Fluc) reporter under the control of native wtLTR (pNLR-Fluc) or modified mLTR3 (pmNLR-Fluc) promoter. **(Right)** Fold-activation of luciferase expression in HEK293T cells co-transfected with pNLR-Fluc or pmNLR-Fluc and empty pcDNA vector (Mock), TALE activator expression constructs (TLT5-7) or Tat and Rev-expression plasmids (Tat/Rev). Luciferase expression in each construct was normalized to cells transfected with Mock control. *Renilla* luciferase expression was used to normalize for transfection efficiency and cell number. Error bars indicate standard deviation of one experiment with three transfection replicates ($n = 3$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; *t*-test sample vs control(Mock)).

We next determined whether engineered mLTR3 promoter affected lentivirus production and maintained expression of suicidal lentivector specific to HIV-1 infected cells. Jurkat T lymphocytes were infected with a non-replicative HIV-1 clone harboring EGFP as reporter for viral infection (NL4-3Δenv-EGFP). Non-infected and HIV-1 infected cells were then transduced with HIV-responsive lentivector (mNLR-E2C) that incorporates a far-red E2-Crimson reporter [400] driven by modified mLTR3 **(Fig. 2.8; Top)**. Additionally, we introduced a WPRE regulatory element [424] downstream of E2C reporter (mNLRW-E2C) to further support lentivector expression **(Fig. 2.8; Top)**. We observed that E2C-positive cells (~17%) derived from mNLR-E2C activation were detected only in GFP-positive HIV-1 infected cells. **(Fig. 2.8; Bottom)**. Furthermore, incorporation of WPRE in mNLW-E2C enhanced lentivector expression (~38% E2C-positive) without affecting specificity to HIV-1 infection. To enforce safety of this approach, delivery of these therapeutic plasmids to target cells should be mediated by integration-deficient lentivirus (IDLV) that avoid genotoxic effects from lentivirus integration into the chromatin however with dramatic reduction of gene expression (see chapter 1.2.1. for details). Still, efficient E2C expression (~26% E2C-positive cells) was detected in HIV-1 infected cells following IDLV delivery of pmNLRW-E2C (Fig. 2.8; Bottom). Briefly, we demonstrate that engineered suicidal lentivector is highly responsive to the presence of active HIV-1 infected cells.

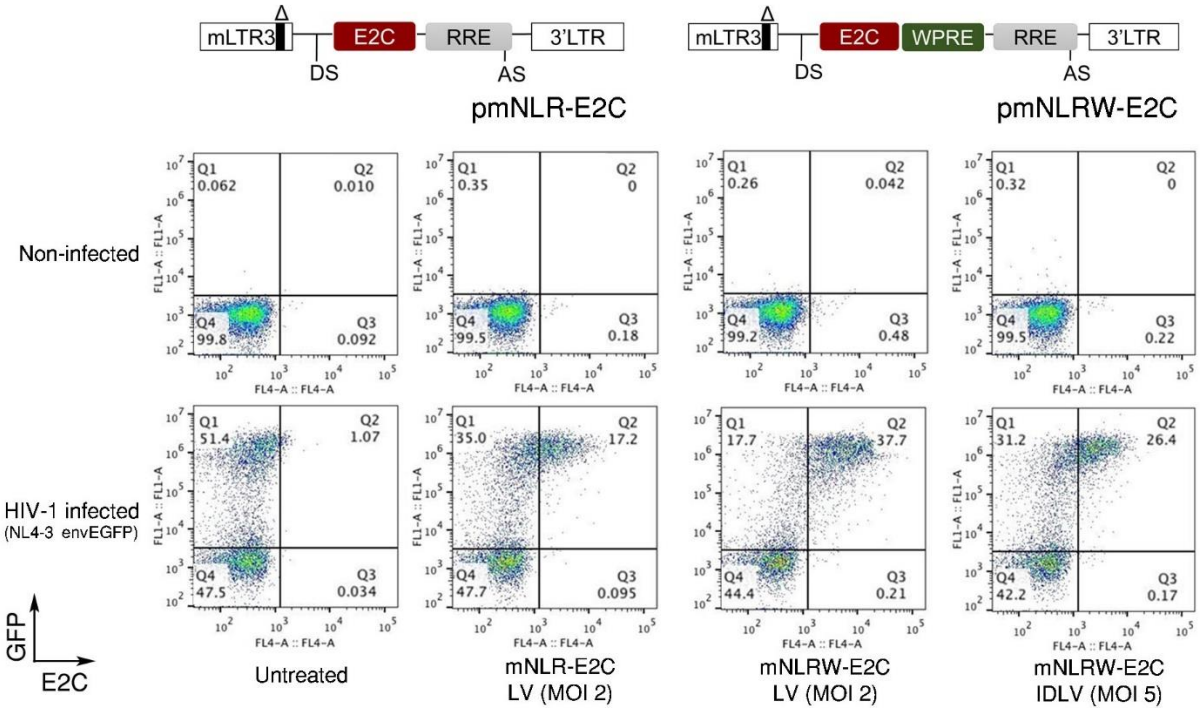

Figure 2.8– Specific gene expression of engineered suicidal lentivector in HIV-1 infected cells. (Top) Schematic representation of mLTR3-driven suicide lentivector incorporating E2Crimson (pmNLR-E2C) reporter. In pmNLRW-E2C, woodchuck hepatitis post-transcriptional regulatory element (WPRE) was incorporated downstream of E2C reporter to further enhance gene expression. **(Bottom)** Percentage of GFP and E2C gene expression of Jurkat cells non-infected or infected at MOI 2 with HIV-1 NL4-3ΔenvEGFP-reporter (HIV-1 infected) followed by transduction of mNLR-E2C lentivirus (LV), mNLRW-E2C lentivirus (LV) or integration-deficient lentivirus (IDLV) at the indicated MOI. Cells were analyzed by flow cytometry 48 hours after transduction. "Untreated" indicates non-transduced with E2C-suicidal vectors.

2.4.7. Suicidal lentivectors with TALE activators specifically eliminate latent HIV-1 cells

To evaluate the selective elimination of HIV latent cells by the proposed strategy, we first compared the suicidal lentivector expression in non-infected Jurkat or HIV latent J-Lat 10.6 cells co-nucleofected with TALE activators (TLT5-7) and pmNLRW-E2C reporter. We show that E2C expression (~11% E2C-positive cells) is only observed in J-Lat 10.6 cells highly expressing GFP, with approximately 11% of J-Lat cells positive for E2-Crimson reporter **(Fig. 2.9A)**. Additionally, we do not detect E2C-positive cells in Jurkat population even in the presence of TLT5-7, indicating that TALE-mediated activation of latent HIV expression is necessary to trigger expression of suicide lentivector.

To promote the elimination of latent HIV population, we explored the potential of toxins derived from pathogenic bacteria, frequently explored for therapeutic applications by inducing programmed cell death of target cells [425]. We tested a different set of potent toxins that mediate cell apoptosis through distinct modes of action: Diptheria toxin A chain from *Corynebacterium diphtheria* and Ricin toxin A chain from *Ricinus communis* block protein synthesis; Anthrax Lethal factor N-terminal from *Bacillus Anthracis* lead to programmed cell death by blocking cell signaling activity; and Streptolysin O from *Streptococcus pyogenes* disrupts cell membrane integrity. J-Lat cells co-nucleofected with TLT5-7 activators and suicide lentivectors incorporating these toxins were analyzed by flow cytometry for by Annexin-V staining of apoptotic cells. Only suicide lentivectors incorporating Diphtheria (~52%) and Ricin (~43%) could lead to apoptosis of J-Lat 10.6 cells **(Fig. 2.9B)**, pointing out that inducible expression of these toxins is sufficient to stimulate cell death.

Finally, we set out to evaluate selective elimination of HIV latent cells. For this, we compared cell death between non-infected Jurkat and HIV latently infected J-Lat 10.6 populations co-nucleofected with TLT5-7 activators and increasing amounts of suicide lentivector driving expression of Diphtheria toxin (pmNLRW-DTA). Significant cell death of both Jurkat and J-Lat is observed with equal amounts of TLT5-7 and pmNLRW-DTA (1 µg)

However, while high-level of J-Lat apoptosis (40-50%) is detected at all pmNLRW-DTA amounts, cell death in Jurkat decreases with lower dose of transfected suicidal lentivector **(Fig. 2.9C)**, indicating that unspecific death of non-infected cells might be caused by leaking expression from excessive plasmid transfection. At the lower amount of lentivector transfected (0.1 µg), we observe a significant difference in cell death between non-infected Jurkat (~10%) and HIV latent J-Lat 10.6 (~50%) populations. Overall, these results demonstrate that TALE-mediated activation of HIV latent cells drive expression of engineered suicidal lentivector and conjugation of both can lead to specific elimination of this population.

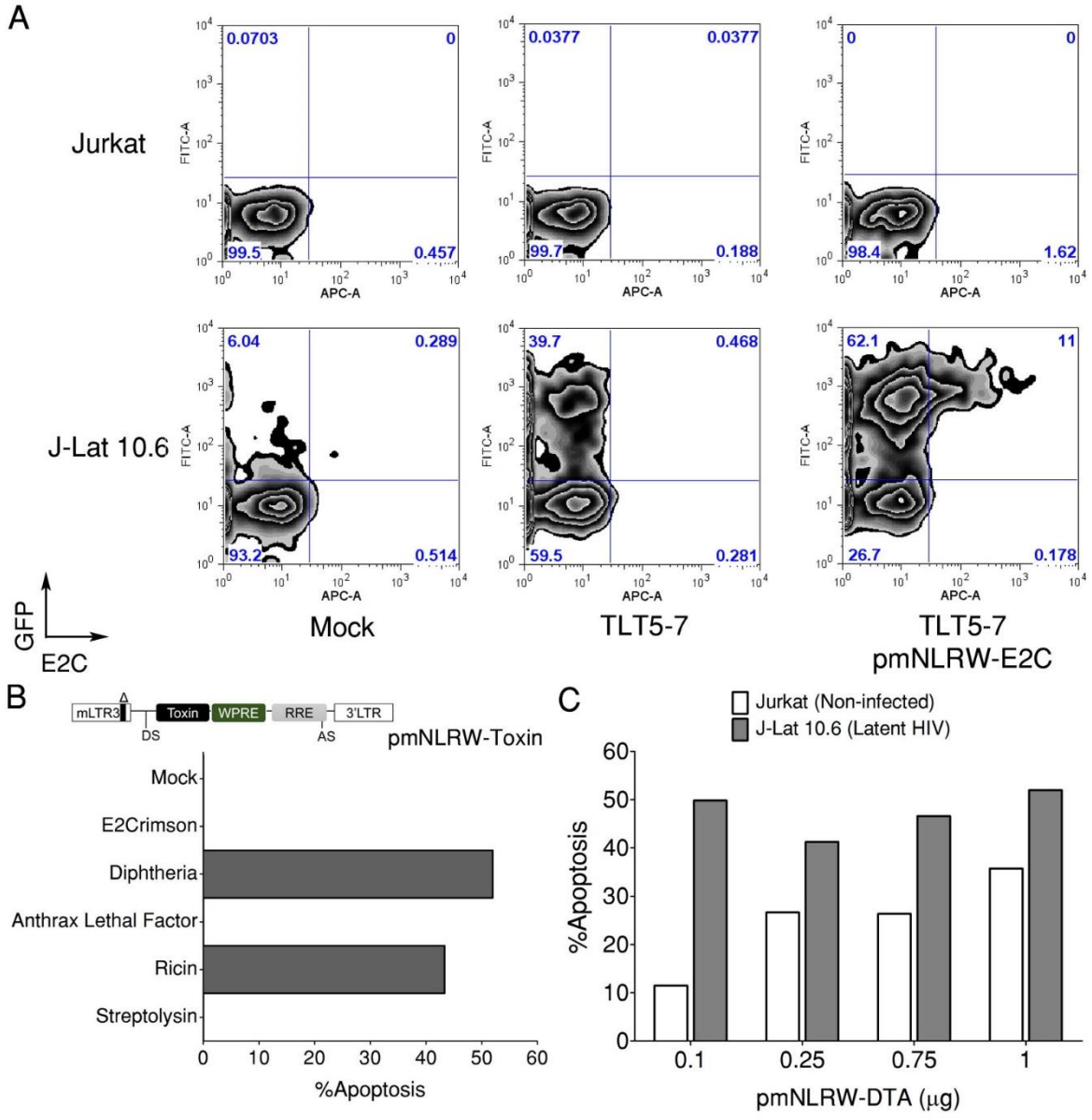

Figure 2.9– Selective elimination of HIV-1 latent cells through conjugation of TALE activators and suicide lentivectors. (A) Percentage of GFP and E2C gene expression of non-infected Jurkat cells or J-Lat 10.6 HIV latent cells nucleofected with pcDNA empty vector (Mock), TALE activators (TLT5-7) alone or with E2C-reporter suicidal lentivector (pmNLRW-E2C). Cells were analyzed by flow cytometry 48 hours after nucleofection. **(B)**

Percentage of apoptotic cells of J-Lat 10.6 population co-nucleofected with pTLT5-7 and HIV suicidal lentivectors incorporating indicated toxin genes (pmNLRW-Toxin). "Mock" indicates cells nucleofected with pcDNA empty vector. "E2Crimson" indicates cells nucleofected with pmNLRW-E2C reporter. Apoptotic cells were detected by flow cytometry analysis following AnnexinV staining 48 hours after cell nucleofection. Percentage of apoptotic cells were normalized to Mock control. (C) Percentage of apoptotic cells of non-infected Jurkat cells or J-Lat 10.6 HIV latent cells co-nucleofected with TLT5-7 activators (1 µg) and increasing amounts of Diphtheria-incorporated suicidal lentivector (pmNLRW-DTA). Cell nucleofection of TLT5-7 and pmNLRW-DTA was supplemented with pcDNA to 2 µg of total DNA Apoptotic cells were detected by flow cytometry analysis following AnnexinV staining at 48 hours after cell nucleofection. Percentage of apoptotic cells were normalized to cells nucleofected with pcDNA vector.

2.5. DISCUSSION

HIV-1 latency is a substantial obstacle facing its eradication. Many approaches have been developed to indirectly activate HIV-1 from persistent cellular reservoirs, typically by altering the transcriptional landscape surrounding the integrated provirus [74,77,79,80,82,83,87,88]. While promising, these strategies have been unable to completely purge all virus from the reservoir and, in some cases, have even been associated with adverse effects, including immune reactions [29,52,78,390]. While targeted nucleases and recombinases have the capacity to excise integrated proviral DNA from infected cells [372–374,383], these tools also have the potential to induce unwanted non-specific DNA breaks, and thus carry a substantial risk of genotoxicity [240]. Conversely, synthetic transcription factors, which can be designed to induce transcription from the native viral promoter, represent a potentially safe and effective genetic alternative for reactivating latent virus in cells.

Here, we designed ten TALE proteins that spanned nearly the entire length of the HIV-1 LTR promoter in order to create activators capable of stimulating HIV-1 transcription. We identified four proteins (TLT5, TLT6, TLT7 and TLT8) that induced viral gene expression in cell line models of HIV latency. Interestingly, we observed that the effectiveness of individual TALEs correlated with their proximity to the TSS, as they targeted a conserved segment of the HIV LTR modulatory region, located upstream of the NF-κB and Sp1 *cis*-regulatory sites, and nearby regulatory elements that contribute to viral transcriptional initiation [407,426]. This data indicates that cooperation between endogenous transcription factors and engineered TALE activators may be an important factor for efficient reactivation of viral gene expression. These TALEs might thus promote transcription in a manner that mimics the natural activity of enhancer-like regulatory proteins, potentially serving as "molecular switches" for reactivation.

We showed that co-transfection of combinations of TALE activators can further increase gene expression, indicating that strategies for mimicking the natural complexity of gene regulation [302,305] are also effective for inducing viral gene expression. Specifically, co-transfection of J-Lat 10.6 cells with TLT5-8 led to similar amounts of HIV-1 expression as those previously reported for compounds such as phytohemagglutinin (PHA), phorbolmyristate acetate (PMA) and prostratin [419]. Moreover, the specificity of our TALE activators could be improved in the future by incorporating recently described chemical- [398,427] or light-inducible [275,428] features that enable spatial and temporal control of HIV-1 expression.

Two reports initially demonstrated that engineered zinc-finger and TALE transcription factors can induce latent HIV expression, albeit with relatively modest efficiencies [429,430]. More recently, several other studies have shown that TALE [431] and CRISPR-Cas9 [432–436] activators can induce robust activation of latent viral expression. Our data correlates with these most recent reports and further indicates the potential of artificial transcription factor technology for eliminating the latent reservoir.

Although the TALE activators used in our study induced efficient HIV-1 expression in J-Lat 10.6 cells, reduced levels of viral gene expression were observed in J-Lat clones containing more repressive transcriptional backgrounds. Specifically, integration of the HIV-1 provirus into condensed regions of heterochromatin can negatively affect viral gene expression by hindering DNA accessibility to key host transcription factors [37,41]. Because histone deacetylases (HDACs) play a central role in maintaining HIV latency by promoting compact chromatin structures around integrated proviral DNA [37], we hypothesized that treating cells with HDAC inhibitors could increase binding site accessibility and further enhance HIV-1 reactivation. We found that combining TALE activators with different classes of HDAC inhibitors led to a significant increase in viral gene expression in J-Lat 6.3 and 10.6 cells comparable to those previously described for other latency-reversing compounds, including PHA and Bryostatin [419]. We also observed that HDAC inhibitor selectivity affects TALE-mediated stimulation of HIV expression, as H-12 (selective for HDAC-1 and -2 only) had no effect on this matter. Our data correlates with previous studies reporting the major contribution of class I HDACs, and more specifically HDAC-3 on the epigenetic regulation of HIV expression [418,437]. This indicates the broad utility of this concept for HIV-1 reactivation, and supports further investigation into the effects of combining HDAC inhibition with TALE-mediated activation in primary cell models of HIV latency [419]. Combining synthetic activators and HDAC inhibition with HAART or nuclease-induced knockout of HIV co-receptors CCR5 and CXCR4 [356] may also prove effective for combating HIV infection.

Additionally, we designed a strategy to enforce elimination of stimulated HIV latent cells by conjugating TALE activators with a HIV-responsive suicide lentivector. We engineered a Tat/Rev-dependent suicide lentivector with a tailored 5'LTR promoter that disrupts TLT5-7 binding and non-specific activation in uninfected cells. This modified vector induced gene expression only in the presence of HIV-1 infected cells, even when delivered as non-integrative IDLV which provides a safety advantage for gene therapeutic applications [138]. We provide evidence that activation of J-Lat HIV latent cells by TLT5-7 is required to induce expression of suicidal lentivector. Conjugation of TLT5-7 and a small dose of suicidal lentivector incorporating a Diphtheria toxin selectively induced efficient cell death of HIV latent cells with minimal damage to healthy uninfected cells, demonstrating the potential of this strategy to safely eliminate HIV reservoirs stimulated by our TALE activator technology.

While TALE activators have the capacity to activate latent HIV-1 transcription, several barriers must be overcome for this technology to be implemented for therapeutic purposes. In particular, due to their highly repetitive nature, lentiviral vector-mediated delivery of TALEs into cells has proven challenging [240,290,438]. Methods for overcoming this limitation are rapidly emerging, including those based on adenoviral [291,439], AAV [275], mRNA [440] and protein-based delivery systems [285,287]. Additionally, recent work has indicated that TALE nucleases could be introduced into cells as mRNA using lentivirus particles containing inactivated reverse transcriptase [441]. However, it remains unknown whether such systems can support *in vivo* delivery to latent resting $CD4^+$ T cells. Although our current study indicates that a specific combination of four TALE activators is optimal for inducing HIV-1 transcriptional activation, current evidence demonstrate that the potency of these activators can be further enhanced to promote single TALE systems [312].

In summary, we demonstrate that TALE activators are effective tools for activating latent HIV expression and their use, alone or in combination with HDAC inhibitors or suicide lentivectors, could pave the way for improved HIV therapies.

2.6. ACKNOWLEDGMENTS

We thank Joel M. Gottesfeld for his generous mentorship. We thank Thomas Gaj for collaborating and supervising TALE design experiments. We thank Cheila Rocha for assisting in the construction of suicidal lentivector. We thank Daria Hazuda and Richard Barnard for kindly providing the HDAC inhibitors. We also thank Daniel Voytas and Adam Bogdanove for the kind gift of the TALEN Golden Gate kit, Maryanne Simurda for kindly providing the pTat

plasmid, Cecília Rodrigues for kindly providing primary mouse anti-β-actin and secondary HRP-conjugated goat anti-mouse IgG antibodies. We thank João Barata for supplying the Nucleofector II device. We thank Jon W. Marsh for providing pNL-GFP-RRE(SA). We thank Wendie Cohick for providing pCAGGS-RTA and Michael Caparon for providing pBAD-SLO. pET-15b LFN-DTA was a gift from John Collier (Addgene plasmid # 11075). FUGW was a gift from David Baltimore (Addgene plasmid # 14883). pTat expression plasmid was kindly provided by Dr. Maryanne Simurda (State University of New York). pRSV-Rev (Addgene plasmid # 12253), pMD2.G (Addgene plasmid # 12259) and psPAX2 (Addgene plasmid # 12260) were a gift from Didier Trono. psPAX2-D64V was a gift from David Rawlings & Andrew Scharenberg (Addgene plasmid # 63586). The following reagents were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, and NIH: pNL4-3 from Dr. Malcolm Martin, pNL4-3Δenv-EGFP from Drs. Haili Zhang, Yan Zhou, and Robert Siliciano, Human rhIL-2 from Dr. Maurice Gately, Hoffmann - La Roche Inc., Jurkat clone E6-1 from Dr. Arthur Wess and J-Lat Full Length clones from Dr. Eric Verdin and SAHA (Vorinostat).

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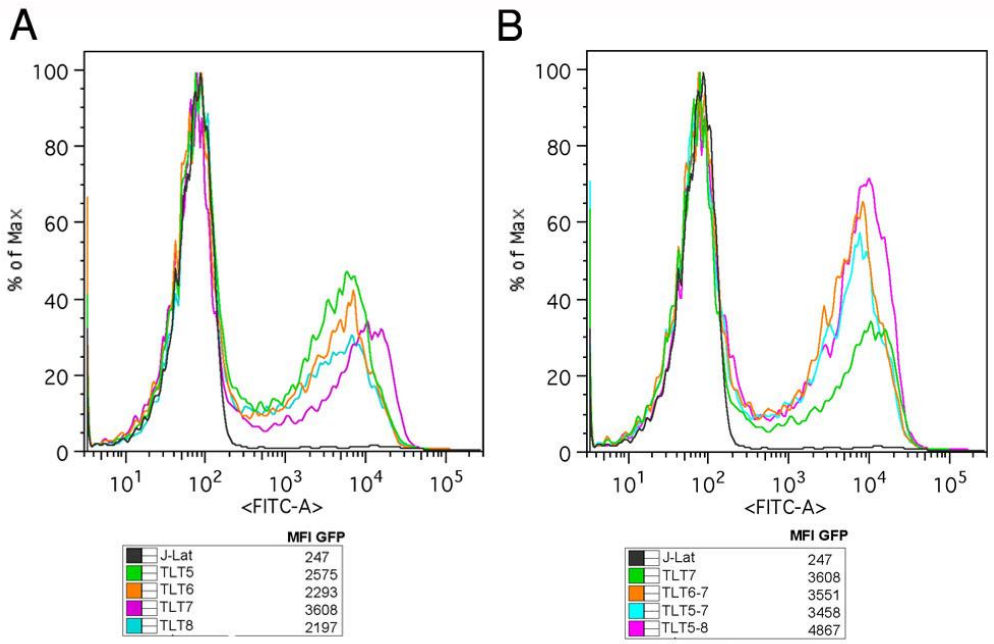
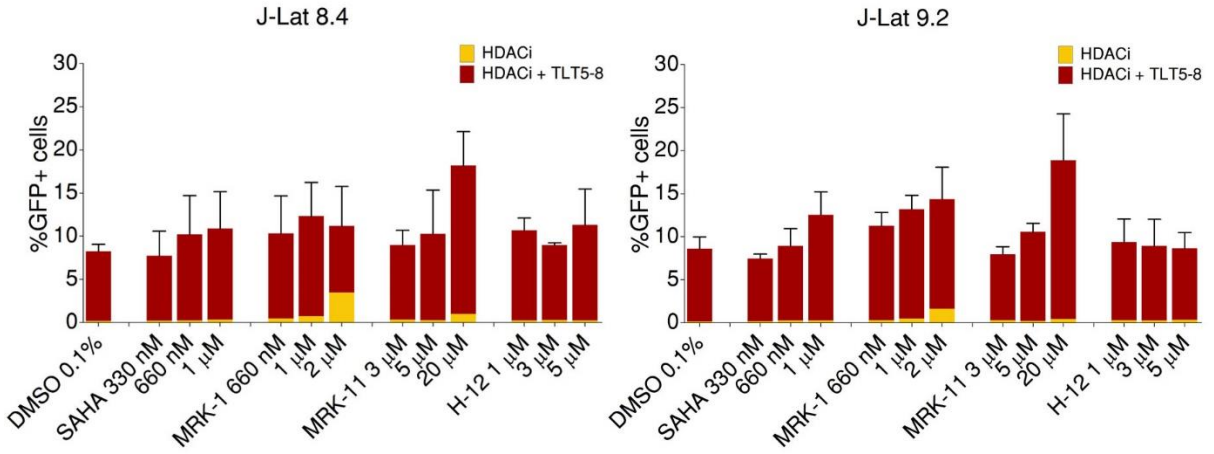
2.7. SUPPLEMENTARY INFORMATION

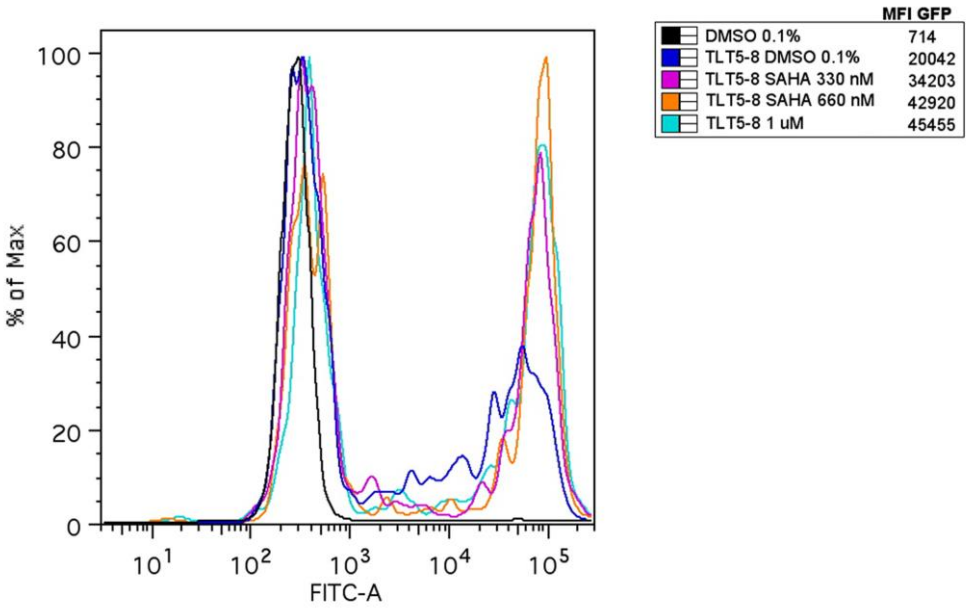
2.7.1. Supplementary Figures



Supplementary Figure S 2.1- Analysis of GFP mean fluorescence intensity in J-Lat 10.6 cells nucleofected with TALE activators. Mean fluorescence intensity (MFI) of GFP expression in J-Lat 10.6 cells after nucleofection with a **(A)** single TALE activator (TLT5, TLT6, TLT7, and TLT8) or **(B)** a combination of TALE activators (TLT7, TLT6-7, TLT5-7, and TLT5-8). MFI was measured by flow cytometry 48 h after nucleofection. "J-Lat" indicates non-transfected J-Lat 10.6 cells. Histograms are representative of a single experiment from three independent replicates.



Supplementary Figure S 2.2 – Evaluation of HIV reactivation in J-Lat 8.4 and 9.2 following co-treatment with TALE activators with class-selective histone deacetylase inhibitors. Percentage of GFP-positive J-Lat 8.4 and 9.2 cells after nucleofection with TLT5-8 expression plasmids and treatment with increasing concentrations of SAHA (330 nM, 660 nM, 1 µM), MRK-1 (660 nM, 1 µM, 2 µM), MRK-11 (3 µM, 5 µM, 20 µM), H-12 (1 µM, 3 µM, 5 µM) or DMSO (0.1%) control for 24 h. GFP-positive cells were measured by flow cytometry 48 h after nucleofection. Error bars indicate standard error of the mean of three independent experiments (n = 3).



Supplementary Figure S 2.3- SAHA increases mean fluorescence intensity in J-Lat cells nucleofected with combinations of TALE activators. Mean fluorescence intensity (MFI) of GFP expression in J-Lat 10.6 cells nucleofected with TALE activators (TLT5-8) and co-treated with SAHA.J-Lat 10.6 cells were nucleofected with TLT5-8 expression plasmids and treated with increasing concentrations of SAHA or DMSO only for 24 h. MFI was measured by flow cytometry 48 h after nucleofection. Histograms are representative of a single experiment from three independent replicates.

2.7.2. Supplementary Tables

Supplementary Table S 2.1- TALE proteins sequences used in this study. TALE N-terminal domain is colored orange. TALE DNA-binding domain is colored blue. RVD residues are shown in red. Nuclear localization signal (NLS) sequence is highlighted grey. VP64 domain is colored green. HA tag is colored purple.

>TLT1-VP64

MAQAASGSPRPPRAKPAPRRRAAQPSDASPAAQVDLRTLGYSQQQQEKIKPKVRSTVAQHHEALVGHGFTHAHIVALSQHPA
ALGTVAVTYQHIITALPEATHEDIVGVGKQWSGARALEALLTDAGELRGPPLQLDTGQLVKIAKRGGVTAMEAVHASRNALTG
AP
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GLTPDQVVAIASHDGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASHDGGKQALETVQRLLAVLCQDH
GLTPDQVVAIASNIGGKQALETVQRLLAVLCQDH
GLTPDQVVAIASHDGGKQALETVQRLLAVLCQDH
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GLTPDQVVAIASNIGGKQALETVQRLLPVLCQDH
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FDLDMLGSDALDDFDLDMLINYPYDVPDYAS

>TLT2-VP64

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FDLDMLGSDALDDFDLDMLINYPYDVPDYAS

>TLT3-VP64

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GLTPDQVVAIASHDGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASHDGGKQALETVQRLLPVLCQDH
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>TLT4-VP64

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AP
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GLTPDQVVAIASHDGGKQALETVQRLLAVLCQDH
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GLTPDQVVAIASHDGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASHDGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASHDGGKQALETVQRLLPVLCQDH
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GLTPDQVVAIASNNGGKQALESIVAQLSRPDPALAALTNDHLVALACLGGRPAMDAVKKGLPHAPELIRRVNRRIGERTSHRVA
DYAQVVRVLEFFQCHSHPAYAFDEAMTQFGMSGQAGQASPKKKRKVGRADALDDFDLDMLGSDALDDFDLDMLGSDALDD
FDLDMLGSDALDDFDLDMLINYPYDVPDYAS

>TLT5-VP64

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ALGTVAVTYQHIITALPEATHEDIVGVGKQWSGARALEALLTDAGELRGPPLQLDTGQLVKIAKRGGVTAMEAVHASRNALTG
AP
LNLTPDQVVAIASNNGGKQALETVQRLLPVLCQDH
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GLTPDQVVAIASHDGGKQALETVQRLLAVLCQDH
GLTPDQVVAIASHDGGKQALETVQRLLAVLCQDH
GLTPDQVVAIASHDGGKQALETVQRLLAVLCQDH
GLTPDQVVAIASNNGGKQALETVQRLLAVLCQDH
GLTPDQVVAIASNIGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNNGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNIGGKQALETVQRLLPVLCQDH

GLTPDQVVAIASNNGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASHDGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNGGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNNGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASHDGGKQALETVQRLLPVLCQDH
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DYAQVVRVLEFFQCHSHPAYAFDEAMTQFGMSGQAGQASPKKKRKVGRADALDDFDLDMLGSDALDDFDLDMLGSDALDD
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>TLT6-VP64
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ALGTVAVTYQHIITALPEATHEDIVGVGKQWSGARALEALLTDAGELRGPPLQLDTGQLVKIAKRGGVTAMEAVHASRNALTG
AP
LNLTPDQVVAIASNNGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASHDGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNIGGKQALETVQRLLAVLCQDH
GLTPDQVVAIASNGGGKQALETVQRLLAVLCQDH
GLTPDQVVAIASHDGGKQALETVQRLLAVLCQDH
GLTPDQVVAIASHDGGKQALETVQRLLAVLCQDH
GLTPDQVVAIASNNGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNNGGKQALETVQRLLPVLCQDH
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GLTPDQVVAIASNNGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNGGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNIGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASHDGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNGGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNIGGKQALESIVAQLSRPDPALAALTNDHLVALACLGGRPAMDAVKKGLPHAPELIRRVNRRIGERTSHRVA
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>TLT7-VP64
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AP
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GLTPDQVVAIASNGGGKQALETVQRLLAVLCQDH
GLTPDQVVAIASNNGGKQALETVQRLLAVLCQDH
GLTPDQVVAIASNIGGKQALETVQRLLAVLCQDH
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GLTPDQVVAIASNNGGKQALETVQRLLPVLCQDH
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GLTPDQVVAIASNGGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNGGGKQALESIVAQLSRPDPALAALTNDHLVALACLGGRPAMDAVKKGLPHAPELIRRVNRRIGERTSHRVA
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>TLT8-VP64

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ALGTVAVTYQHIITALPEATHEDIVGVGKQWSGARALEALLTDAGELRGPPLQLDTGQLVKIAKRGGVTAMEAVHASRNALTG
AP
LNLTPDQVVAIASNGGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNGGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASHDGGKQALETVQRLLAVLCQDH
GLTPDQVVAIASHDGGKQALETVQRLLAVLCQDH
GLTPDQVVAIASNNGGKQALETVQRLLAVLCQDH
GLTPDQVVAIASHDGGKQALETVQRLLAVLCQDH
GLTPDQVVAIASNGGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNNGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNNGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNNGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNNGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNIGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASHDGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNGGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNGGGKQALESIVAQLSRPDPALAALTNDHLVALACLGGRPAMDAVKKGLPHAPELIRRVNRRIGERTSHRVA
DYAQVVRVLEFFQCHSHPAYAFDEAMTQFGMSGQAGQASPKKKRKVGRADALDDFDLDMLGSDALDDFDLDMLGSDALDD
FDLDMLGSDALDDFDLDMLINYPYDVPDYAS

>TLT9-VP64

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ALGTVAVTYQHIITALPEATHEDIVGVGKQWSGARALEALLTDAGELRGPPLQLDTGQLVKIAKRGGVTAMEAVHASRNALTG
AP
LNLTPDQVVAIASNNGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNNGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASHDGGKQALETVQRLLAVLCQDH
GLTPDQVVAIASNNGGKQALETVQRLLAVLCQDH
GLTPDQVVAIASNIGGKQALETVQRLLAVLCQDH
GLTPDQVVAIASNNGGKQALETVQRLLAVLCQDH
GLTPDQVVAIASHDGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASHDGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASHDGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNGGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASHDGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNIGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNNGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNIGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNGGGKQALESIVAQLSRPDPALAALTNDHLVALACLGGRPAMDAVKKGLPHAPELIRRVNRRIGERTSHRVA
DYAQVVRVLEFFQCHSHPAYAFDEAMTQFGMSGQAGQASPKKKRKVGRADALDDFDLDMLGSDALDDFDLDMLGSDALDD
FDLDMLGSDALDDFDLDMLINYPYDVPDYAS

>TLT10-VP64

MAQAASGSPRPPRAKPAPRRRAAQPSDASPAAQVDLRTLGYSQQQQEKIKPKVRSTVAQHHEALVGHGFTHAHIVALSQHPA
ALGTVAVTYQHIITALPEATHEDIVGVGKQWSGARALEALLTDAGELRGPPLQLDTGQLVKIAKRGGVTAMEAVHASRNALTG
AP
LNLTPDQVVAIASNGGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNIGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNGGGKQALETVQRLLAVLCQDH
GLTPDQVVAIASNGGGKQALETVQRLLAVLCQDH
GLTPDQVVAIASNNGGKQALETVQRLLAVLCQDH
GLTPDQVVAIASNIGGKQALETVQRLLAVLCQDH
GLTPDQVVAIASNNGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNNGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASHDGGKQALETVQRLLPVLCQDH

GLTPDQVVAIASNGGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNGGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNIGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNIGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASNNGGKQALETVQRLLPVLCQDH
GLTPDQVVAIASHDGGKQALESIVAQLSRPDPALAALTNDHLVALACLGGRPAMDAVKKGLPHAPELIRRVNRRIGERTSHRVA
DYAQVVRVLEFFQCHSHPAYAFDEAMTQFGMSGQAGQASPKKKRKVGRADALDDFDLDMLGSDALDDFDLDMLGSDALDD
FDLDMLGSDALDDFDLDMLINYPYDVPDYAS

Supplementary Table S 2.2- Primer sequences for the construction of the luciferase reporter plasmids used in this study. TALE binding sites are underlined. Restriction sites are in bold.

>5' TALE-Luc-TLT1
ACTGCTAT**CTCGAG**TACCACACACAAGGCTTAGCGTACCACACACAAGGCTTAGCGTACCACACA
CAAGGCTTAGCGTACCACACACAAGGCTTAGCGATCTGCGATCTAAGTAAGCT

>5' TALE-Luc-TLT2
ACTGCTAT**CTCGAG**TGACCTTTGGATGGTGTAGCGTGACCTTTGGATGGTGTAGCGTGACCTTTGG
ATGGTGTAGCGTGACCTTTGGATGGTGTAGCGATCTGCGATCTAAGTAAGCT

>5' TALE-Luc-TLT3
ACTGCTAT**CTCGAG**TTGTTACACCCTGTGATAGCGTTGTTACACCCTGTGATAGCGTTGTTACACCC
TGTGATAGCGTTGTTACACCCTGTGATAGCGATCTGCGATCTAAGTAAGCT

>5' TALE-Luc-TLT4
ACTGCTAT**CTCGAG**TCACATGGCCCGAGAGTAGCGTCACATGGCCCGAGAGTAGCGTCACATGGC
CCGAGAGTAGCGTCACATGGCCCGAGAGTAGCGATCTGCGATCTAAGTAAGCT

>5' TALE-Luc-TLT5
ACTGCTAT**CTCGAG**TGGCCCGAGAGCTGCATAGCGTGGCCCGAGAGCTGCATAGCGTGGCCCGAG
AGCTGCATAGCGTGGCCCGAGAGCTGCATAGCGATCTGCGATCTAAGTAAGCT

>5' TALE-Luc-TLT6
ACTGCTAT**CTCGAG**TGCATCCGGAGTACTATAGCGTGCATCCGGAGTACTATAGCGTGCATCCGGA
GTACTATAGCGTGCATCCGGAGTACTATAGCGATCTGCGATCTAAGTAAGCT

>5' TALE-Luc-TLT7
ACTGCTAT**CTCGAG**TGCTGACATCGAGCTTTAGCGTGCTGACATCGAGCTTTAGCGTGCTGACATC
GAGCTTTAGCGTGCTGACATCGAGCTTTAGCGATCTGCGATCTAAGTAAGCT

>5' TALE-Luc-TLT8
ACTGCTAT**CTCGAG**TTTCCGCTGGGGACTTTAGCGTTTCCGCTGGGGACTTTAGCGTTTCCGCTGGG
GACTTTAGCGTTTCCGCTGGGGACTTTAGCGATCTGCGATCTAAGTAAGCT

>5' TALE-Luc-TLT9
ACTGCTAT**CTCGAG**TGGCGAGCCCTCAGATTAGCGTGGCGAGCCCTCAGATTAGCGTGGCGAGCC
CTCAGATTAGCGTGGCGAGCCCTCAGATTAGCGATCTGCGATCTAAGTAAGCT

>5' TALE-Luc-TLT10
ACTGCTAT**CTCGAG**TTATTGAGGCTTAAGCTAGCGTTATTGAGGCTTAAGCTAGCGTTATTGAGGC
TTAAGCTAGCGTTATTGAGGCTTAAGCTAGCGATCTGCGATCTAAGTAAGCT

>3' Luc-Rev
CGTTTTCCCGGTACCAGAT

>5' LTR-Fwd
CG**ACGCGT**TGGAAGGGCTAATTTGGTCCCA

>3' LTR-Rev
CTA**GCTAGC**TTGAGCACTCAAGGCAAGCTTTATTG

Supplementary Table S 2.3- Modified 5'LTR (mLTR) sequences tested in this study. TALE binding sites are underlined. LTR substituted sequence are highlighted in yellow.

>wtLTR
TGGAAGGGCTAATTTGGTCCCAAAAAAGACAAGAGATCCTTGATCTGTGGATCTACCACACACAAGGCTACTTCCCTGATT
GGCAGAACTACACACCAGGGCCAGGGATCAGATATCCACTGACCTTTGGATGGTGCTTCAAGTTAGTACCAGTTGAACCA
GAGCAAGTAGAAGAGGCCAATGAAGGAGAGAACAACAGCTTGTTACACCCTATGAGCCAGCATGGGATGGAGGACCCG
GAGGGAGAAGTATTAGTGTGGAAGTTTGACAGCCTCCTAGCATTTCGTCACATGGCCCGAGAGCTGCATCCGGAGTACTA
CAAAGACTGCTGACATCGAGCTTTCTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGTGTGGCCTGGGCGGGACTG
GGGAGTGGCGAGCCCTCAGATGCTACATATAAGCAGCTGCTTTTTGCCTGTACTGGGTCTCTCTGGTTAGACCAGATCTGA
GCCTGGGAGCTCTCTGGCTAACTAGGGAACCCACTGCTTAAGCCTCAATAAAGCTTGCCTTGAGTGCTCAA

>mLTR1
CTTTCTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGTGTGGCCTGGGCGGGACTGGGGAGTGGCGAGCCCTCAG
ATGCTACATATAAGCAGCTGCTTTTTGCCTGTACTGGGTCTCTCTGGTTAGACCAGATCTGAGCCTGGGAGCTCTCTGGCTA
ACTAGGGAACCCACTGCTTAAGCCTCAATAAAGCTTGCCTTGAGTGCTCAA

>mLTR2
TGGAAGGGCTAATTTGTCCCAAAAAAGACAAGAGATCCTTGATCTGTGGATCTACCACACACAAGGCTACTTCCTGATTGG
CAGAACTACACACCAGGGCCAGGGATCAGATATCCACTGACCTTTGGATGGTGCTTCAAGTTAGTACCAGTTGAACCAGA
GCAAGTAGAAGAGGCCAATGAAGGAGAGAACAACAGCTTGTTACACCCTATGAGCCAGCATGGGATGGAGGACCCGGA
GGGAGAAGTATTAGTGTGGAAGTTTGACAGCCTCCTAGCATTTCGTCACATGGCCCGAGAGCTGCATGCATATGGCATGC
CTCGAGAAGCTTACCGGTATTTCTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGTGTGGCCTGGGCGGGACTGGG
GAGTGGCGAGCCCTCAGATGCTGCATATAAGCAGCTGCTTTTTGCCTGTACTGGGTCTCTCTGGTTAGACCAGATCTGAGC
CTGGGAGCTCTCTGGCTAACTAGGGAACCCACTGCTTAAGCCTCAATAAAGCTTGCCTTGAGTGCTCAA

>mLTR3
TGGAAGGGCTAATTTGGTCCCAAAAAAGACAAGAGATCCTTGATCTGTGGATCTACCACACACAAGGCTACTTCCCTGATT
GGCAGAACTACACACCAGGGCCAGGGATCAGATATCCACTGACCTTTGGATGGTGCTTCAAGTTAGTACCAGTTGAACCA
GAGCAAGTAGAAGAGGCCAATGAAGGAGAGAACAACAGCTTGTTACACCCTATGAGCCAGCATGGGATGGAGGACCCG
GAGGGAGAAGTATTAGTGTGGAAGTTTGACAGCGCCATATGGCATGCCTCGAGAAGCTTACCGGTATGCCATATGGCATG
CCTCGAGAAGCTTACCGGTATTTTCTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGCGTGGCCTGGGCGGGACTG
GGGAGTGGCGAGCCCTCAGATGCTACATATAAGCAGCTGCTTTTTGCCTGTACTGGGTCTCTCTGGTTAGACCAGATCTGA
GCCTGGGAGCTCTCTGGCTAACTAGGGAACCCACTGCTTAAGCCTCAATAAAGCTTGCCTTGAGTGCTCAA

>mLTR4
TGGAAGGGCTAATTTGGTCCCAAAAAAGACAAGAGATCCTTGATCTGTGGATCTACCACACACAAGGCTACTTCCCTGATT
GGCAGAACTACACACCAGGGCCAGGGATCAGATATCCACTGACCTTTGGATGGTGCTTCAAGTTAGTACCAGTTGAACCA
GAGCAAGTAGAAGAGGCCAATGAAGGAGAGAACAACAGCTTGTTACACCCTATGAGCCAGCATGGGATGGAGGACCCG
GAGGGAGAAGTATTAGTGTGGAAGTTTGACAGCTTTCTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGCGTGGCC
TGGGCGGGACTGGGGAGTGGCGAGCCCTCAGATGCTACATATAAGCAGCTGCTTTTTGCCTGTACTGGGTCTCTCTGGTT
AGACCAGATCTGAGCCTGGGAGCTCTCTGGCTAACTAGGGAACCCACTGCTTAAGCCTCAATAAAGCTTGCCTTGAGTGCT
CAA

>mLTR5
TGGAAGGGCTAATTTGGTCCCAAAAAAGACAAGAGATCCTTGATCTGTGGATCTACCACACACAAGGCTACTTCCCTGATT
GGCAGAACTACACACCAGGGTCAGGGATCAGATATCCACTGACCTTTGGATGGTGCTTCAAGTTAGTACCAGTTGAACCA
GAGCAAGTAGAAGAGGCCAATGAAGGAGAGAACAACAGCTTGTTACACCCTATGAGCCAGCATGGGATGGAGGACCCG
GAGGGAGAAGTATTAGTGTGGAAGTTTGACAGCGCATGTGGGTCTCTCTGGTTAGACCAGATCTGAGCCTGGGAGCTCTC
TGGCTAACTAGGGAACCCAGCTTTCTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGTGTGGCCTGGGCGGGACTG
GGGAGTGGCGAGCCCTCAGATGCTACATATAAGCAGCTGCTTTTTGCCTGTACTGGGTCTCTCTGGTTAGACCAGATCTGA
GCCTGGGAGCTCTCTGGCTAACTAGGGAACCCACTGCTTAAGCCTCAATAAAGCTTGCCTTGAGTGCTCAA

Supplementary Table S 2.4- Primer sequences for the construction of the modified luciferase reporter plasmids used in this study. Restriction sites are in bold.

>5' U3-Fwd
CG**ACGCGTAGGCCT**CCACCTGGGTC

>3' R-Rev
CTA**GCTAGC**TTGAGCACTCAAGGCAAGCTTTATTG

>5' -115 U3-Fwd
TTTCTACAAGGGACTTTCCGCTGG

>3' -455 U3-Rev
CCAGCGGAAAGTCCCTTGTAGAAAGCCTTTCCTGGTGTAAGTAGAACTGG

>3' -147/-115 U3-Rev
CCAGCGGAAAGTCCCTTGTAGAAATACCGGTAAGCTTCTCGAGGCATGCCATATGCATGCAGCTCT
CGGGCCAT

>3' -183/-115 U3-Rev
CCAGCGGAAAGTCCCTTGTAGAAAATACCGGTAAGCTTCTCGAGGCATGCCATATGGCATACCGGT
AAGCTTCTCGAGGCATGCCATATGGCGCTGTCAAACTTCCACACTAATACTTCTCC

>3' -183 U3-Rev
CCAGCGGAAAGTCCCTTGTAGAAAGCTGTCAAACTTCCACACTAATACTTCTCC

>3' -183/-115 TAR U3-Rev
CCAGCGGAAAGTCCCTTGTAGAAAGCTGGGTTCCCTAGTTAGCCAGAGAGCTCCCAGGCTCAGATC
TGGTCTAACCAGAGAGACCCACATGCGCTGTCAAACTTCCACACTAATACTTCTCC

>5' U5-Fwd
CAATAAAGCTTGCCTTGAGTGCTCAAAGTAGTGTGTGCCCGTCTGTT

>3' U5-Rev
TTCCGCGGCCGCTATGGCCGAC**GTCGACGGATCC**GCCCGGGTGCTACTACTAATGC

Supplementary Table S 2.5- Primer sequences for the construction of the HIV suicidal lentivectors used in this study. Restriction sites are in bold.

>5' FLuc-Fwd
ACGC**GTCGAC**ATGGAAGACGCCAAAAACATAAAGAAAGG

>3' FLuc-Rev
CTAG**TCTAGA**TTACACGGCGATCTTTCCGCC

>5' E2C-Fwd
CGCGGATCC**GTCGAC**GTCGCCACCATGGATAGCACT

>3' E2C-Rev
CTAG**TCTAGA**GCTAGCGCTACTGGAACAGGTGGTGGC

>5' WPRE-Fwd
TCCAGTAGCGCTAGCTCTAGAATTCGATATCAAGCTTATCGATAATCAACCTCT

>3' WPRE-Rev
GGGTACACTTACCTGGTACCTGCGGGGAGGCGGC

>5' DTA-Fwd
ACGC**GTCGAC**ATGGGCGCTGATGATGTTGTTG

>3' DTA-Rev
CTAG**TCTAGA**TTAGAGCTTTAAATCTCTGTAGGTAGTTTGTCC

>5' LFN-Fwd
ACGC**GTCGAC**ATGGCGGGCGGTCATGG

>3' LFN-Rev
CTAG**TCTAGA**TCAGGATAGATTTATTTCTTGTTCGTTAAATTTATCCATGTAATTAAAAG

>5' RTA-Fwd
ACGC**GTCGAC**ATGATCTTCCCCAAGCAGTACCCTATC

>3' RTA-Rev
CTAG**TCTAGA**TCAAAACTGGCTAGAGGGGGG

>5' SLO-Fwd
ACGC**GTCGAC**ATGCATCATCATCATCATCATGAATCGAACAAACA

>3' SLO-Rev
CTAG**TCTAGA**CTACTTATAAGTAATCGAACCATATGGGCTCA

Supplementary Table S 2.6- IC50 values for HDAC inhibitors used in this study. Values are presented relative to HDACs 1-3, 6 and 8. Data provided by Merck Research Laboratories.

	HDAC IC50 (µM)				
	HDAC1	HDAC2	HDAC3	HDAC6	HDAC8
SAHA	0.004	0.011	0.003	0.002	1
MRK-1	0.0129	0.0965	0.0449	5.4	0.030
MRK-11	0.0789	0.4597	0.1546	0.0057	0.0045
H1/2	0.0083	0.0465	4.8	>50*	>50*

*Highest concentration tested

Supplementary Table S2.7. Sequence conservation of the TALE transcription factor binding sites across HIV-1 subtype B strains. Data based on 2014 edition of the HIV Sequence Database (http://hiv-web.lanl.gov). Dashes indicate sequence identity between subtype strains. Dots indicate gaps in the HIV genome sequence.

	TLT5	**TLT6**	**TLT7**	**TLT8**
HIV strain	TGGCCCGAGAGCTGCA	TGCATCCGGAGTACTA	TGCTGACATCGAGCTT	TTTCCGCTGGGGACTT
B.FR.83.HXB2_LAI_IIIB_BRU.K03455	----------	----------T	----------	----------
B.AU.86.MBC200.AF042100	----------	----------	--A----C--------	----------
B.AU.87.MBC925.AF042101	----------	----------	--------G-------	----------
B.AU.95.C24.AF538304	-----AA---------	----------	---------T------	----------
B.BR.02.02BR011.DQ358809	----------AAA--	AA----------	--------G----T--	----------
B.CN.02.02HNsc11.DQ007903	-----A----------	----------	----------T--	------T---------
B.CN.05.05CNHB_hp3.DQ990880	----------AAA--	AA-----T----T---	--------CT-----A	----------
B.ES.89.U61.DQ854716	A----------A--	-A----------	----------	----------
B.GB.83.CAM1.D10112	----------AA---	A----------T---	----------	----------
B.GB.86.GB8_46R.AJ271445	-------C--A-C---	C----------TT--	----------GAG--	----------
B.GB.x.MANC.U23487	----------	----------	--------GGC-----	--------A-------
B.JP.00.DR2508.AB289588	-----A----------	----------T-----	----------.AG--	----------
B.JP.04.DR5913.AB480696	-------C-----A--	-A----------TT--	----------	----------
B.JP.05.DR6538.AB287363	A----------AA--	AA----------TT--	----------T--	----------
B.JP.98.DR1120.AB480698	----------AAA--	AA----------TT--	----------A--	----------
B.KR.03.03KGS5.JQ316132	----------AAA--	AA----------	----------AAC	----------
B.KR.04.04LHS6.AY839827	.....----------	----------	--------C-------	----------
B.KR.05.05YJN2.JQ316134	-A---------AAA--	AA----------	--------CT------	----------
B.NL.96.H434_42_A1.AY970948	----------	----------	----------	----------
B.TW.94.TWCYS_LM49.AF086817	GA----------A--	-A----------	--------Y-------	--------.T-------
B.US.00.ES1_20.EF363123	----------	----------T	----------	----------
B.US.01.REJO_TF1.JN944911	----------AA---	A----------T	--------C-------	--------.-------
B.US.04.ES4_24.EF363124	----------	----------	----------	----------
B.US.06.CH106_TF1.JN944897	-A---------AAA--	AA----------T---	----------	----------

```
B.US.09.C1P.GU733713              -A---------AAA--   AA----------T  --------C--CT--C  ----------------
B.US.10.VC1.JN397364              -A---------AAA--   AA----------  ----------------  ----------------
B.US.11.CP10_3A.KF384798          ----------------   ----------T-----  --------G-------  ----------------
B.US.83.5018_83.AY835777          ----------------   ----------------  ----------------  ----------------
B.US.84.5019_84.AY835779          ----------------   ----------------  ----------------  ----------------
B.US.85.5077_85.AY835769          ----------------   ----------------  ----------------  ----------------
B.US.86.5084_86.AY835775          ----------------   ----------------  ----------------  ----------------
B.US.87.5113_87.AY835758          ----------------   ----------------  ----------------  ----------------
B.US.88.5160_88.AY835763          ----------------   ----------------  ----------------  ----------------
B.US.89.P896_89_6.U39362          ----------------   ---------------T  --------CT-----A  ----------------
B.US.90.WEAU160_GHOSH.U21135      -A--------------   ---------------T  -------------T--  -----A----------
B.US.91.5048_91.AY835761          ----------------   ----------------  ----------------  ----------------
B.US.94.5082_94.AY835773          ----------------   ----------------  ----------------  ----------------
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CHAPTER III

Protein delivery of cell-penetrating zinc-finger activators to stimulate latent HIV-1 expression

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3.1. ABSTRACT

Despite all efforts to develop an effective treatment capable of eradicating HIV-1 from the infected patient, a cure has not been achieved up to this day. While highly active anti-retroviral therapy (HAART) targets viral replication, latent non-replicative HIV-1 is able to escape this treatment. Strategies that could reactivate HIV-1 latent cells and expose cellular reservoirs to combined HAART and host immune system have been proposed for HIV-1 eradication. Pharmacological approaches tested so far were unsuccessful and led to severe side-effects in the patients mainly due to systemic immune activation. Here, we present an alternative approach through protein delivery of cell-penetrating zinc-finger activators (CPP-ZFA) for direct translocation and stimulation of HIV latent cells. CysHis2 zinc-fingers in fusion with VP64 activation domain were engineered to specifically target the HIV 5'LTR promoter and induce viral transcription. A single zinc-finger activator (PBS1-VP64) was capable of reactivating viral expression from a cell model of HIV latency. Following protein delivery, this CPP-ZFA was able to efficiently transfect and stimulate HIV latent expression. When fused with multiple positively charged nuclear localization signal (NLS) repeats, the entry capability and reactivation potency of this artificial transcription factor were dramatically increased. On the other hand, we show that short-term protein delivery of a single CPP-ZFA is able to efficiently induce sustained gene expression in HIV-1 latent cells. Our work demonstrates that protein delivery of zinc-finger activators is a potential and safer alternative to promote reactivation of HIV-1 latent cells

Keywords: HIV latency / Zinc-finger activators / Cell-penetrating peptides / Protein delivery

3.2. INTRODUCTION

The HIV-1 infection global pandemic is still one of the major concerns in public health. Development of antiretroviral drugs targeting key steps of HIV-1 replication dramatically inhibited progression of infection while extending lifespan of infected patients [30–32]. However, antiretroviral therapy does not provide a cure to patients, requiring continuous administration throughout their life course and often associated with toxic side effects [29]. Inability of antiretroviral therapy to eradicate HIV infection is generally due to existence of transcriptionally silent HIV reservoirs that persist in patients but re-emerge once the treatment is interrupted [36]. HIV latent reservoirs can be found mostly in resting CD4+T cells that harbor integrated provirus but suppress viral expression due to its non-permissive transcriptional environment [37]. The absence of viral replication allows these latent reservoirs to escape antiretroviral action or host immune response, representing the major barrier towards a cure for HIV infection.

Considering the mechanisms behind HIV latency, a "shock and kill" strategy has been proposed to eradicate latent reservoirs. This approach attempts to activate latent HIV expression and expose reservoirs to the immune response or cytopathic effects caused by viral replication to clear the infected cells [72]. Initial attempts were based on pharmacological drugs that manipulated the transcriptional landscape surrounding latent integrated provirus by engaging cell signaling pathways, using monoclonal antibodies, [74] cytokines [76,77] or agonists of protein kinase C (PCK) activation [79–81]; or altering epigenetic landmarks through inhibitors of histone deacetylases (HDACs) [82,83] or DNA methyltransferases [62,63]. Despite evidence that these drugs can induce latent viral expression, none of these strategies were able to significantly reduce HIV reservoirs, and some of them were associated with undesirable and toxic generalized immune activation [42,52], highlighting the need for alternative strategies to induce latent HIV expression.

Despite their silent nature that limits development of targeted strategies, HIV latent cells share a trait marker resultant from the provirus integration, that although silenced leaves a scar in the cell genome. Emergence of DNA-binding domains, including zinc-fingers [243], transcription activator-like effector (TALE) [391] proteins, or more recently the CRISPR-Cas9 system [251] have enabled the generation of genome engineering tools that can directly target specific sites in the human genome and modify its sequence or modulate the transcriptional environment [278]. Engineered zinc-finger domains were the first to be successfully implemented for genome engineering applications [242]. The best characterized Cys2His2

motif is composed of approximately 30 amino acids arranged in a ββα conformation, with residues within α-helix at position -1, 3 and 6 making contact with three base-pairs (triplets) [253]. Due to their versatility, zinc-fingers were assembled to effector domains and repurposed for generation of targeted transcriptional activators or repressors [260,442], epigenetic modulators [326,443], and site-directed nucleases [444,445] or recombinases [446,447].

Zinc-finger engineering studies have also gained relevance to develop antiviral strategies targeting HIV infection. Development of site-directed zinc-finger nucleases have proven effective at inhibiting viral infection by directly removing integrated provirus from infected cells [372–374] or turning autologous cells resistant to HIV entry through knockout of major HIV co-receptors [289,357,362,364], the latter culminating in clinical studies [358]. Inhibition of HIV transcription through zinc-finger based transcriptional repressors [343–346] have also proven effective at shutting down HIV replication.

Given the nature of silent HIV reservoirs, targeted transcription activators have a particular interest to reverse HIV-1 latency. We [448] and others [429–432,434–436,449] have demonstrated the potential of site-directed artificial transcription factors to recognize the HIV promoter and stimulate latent HIV expression, providing a targeted strategy for the "shock and kill" approach. Interestingly, zinc-fingers were found to possess a natural ability to cross the anionic cellular membrane, owing to the positive net charge involving these DNA-binding domains [243]. This cell penetrating capacity grants zinc-fingers a great potential for direct protein delivery that could be useful to transport protein cargo [450] but also make precise genome modifications [263,264].

Herein, we describe a novel approach to target HIV latent cells by direct protein delivery of cell-penetrating zinc-finger activators (CPP-ZFA). We show proof-of-concept of an engineered CPP-ZFA that can directly penetrate HIV latent cells without the need of any carrier, and target the 5'LTR promoter to induce viral expression. Our work demonstrate that engineered zinc-finger activators are a promising tool to target HIV latency and its innate cell-penetrating ability could overcome some of the limitations associated with delivery of these artificial transcription factors to target cells.

3.3. MATERIALS AND METHODS

3.3.1. Zinc-finger activator constructs

Design of HIV-targeted polydactyl zinc-fingers activators (ZFA) was performed at Zinc-finger Tools web server (http://www.zincfingertools.org) [451]. Zinc-finger arrays were generated by modular assembly [452]. ANN [256], GNN [254,255] or CNN [257] binding zinc-finger modules were isolated through XmaI/SpeI digestion and cloned into AgeI/SpeI restriction sites of pSCV [452] vector until generation of 4-finger or 6-finger arrays. ZFAs were generated by cloning of the zinc-finger arrays into XhoI/SpeI restriction sites of pAart-VP64 (pAart6) [453] to generate ZFA constructs pZLT4A through pZLT6B and pAart4. Previously designed HIV-targeted zinc-fingers repressors [344,346] were digested and cloned into XhoI/SpeI restriction sites of pAart6 to generate ZFA expression constructs pHLTR1 through pPBS3.Correct construction of each plasmid was verified by sequence analysis (**Table S3.1**).

pET28b-PBS1-VP64 was generated by PCR amplifying PBS1-VP64 from pPBS1 using primers 5’ ZF-Fwd 3’ VP64-Rev and cloning into NdeI/SacI restriction sites of pET28b-CCR5R-1NLS [264]. pET28b-3NLS-PBS1-VP64 and pET28b-3NLS-Aart6-VP64 were generated by respectively PCR amplifying PBS1-VP64 from pPBS1 and Aart6-VP64 from pAart6 using primers 5’ 3NLS-ZF-Fwd and 3’ VP64-Rev. PCR products were digested with SalI and SacI and cloned into XhoI/SacI restriction sites of pET28b-CCR5R-1NLS. Correct construction of each plasmid was verified by sequence analysis (**Table S3.2**). Primer sequences are provided in **Table S3.3**.

3.3.2. Luciferase reporter constructs

The pZFA luciferase reporter vectors were constructed through PCR by amplifying the luciferase gene from pGL3-Basic (Promega, Madison, WI, USA) using the primers 5’ ZF-Luc-ZLT4A through PBS3, which contained four direct repeats of each zinc-finger binding site and 3’ Luc-Rev. PCR products were digested and cloned into the XhoI/SphI restriction sites of pGL3-Basic to generate pGL3-ZF-ZLT4A through PBS3. The HIV-1 LTR-PBS reporter plasmid was constructed by PCR amplifying the 5’LTR (U3-R-U5) promoter and PBS region from pNL4-3 (NIH AIDS Reagents) [399] using the primers 5’ LTR-PBS-Fwd and 3’ LTR-PBS-Rev. PCR product was digested and cloned into the MluI/NheI restriction sites of pGL3-Basic to generate pGL3-LTR-PBS. Primer sequences are provided in **Table S3.3**.

3.3.3. Cell culture

Human embryonic kidney 293T (HEK293T) (American Type Culture Collection; ATCC) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 2 mM L-glutamine and 1% (v/v) antibiotic-antimycotic (Anti-Anti; Gibco, Carlsbad, CA, USA). Jurkat E6-1 and J-Lat clones (NIH AIDS Reagents) were cultured in RPMI-1640 medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% (v/v) FBS, 2 mM L-glutamine and 1% (v/v) Anti-Anti. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

3.3.4. Luciferase assays

Luciferase assays were performed as previously described [448]. Briefly, HEK293T cells were seeded onto 96-well plates at a density of 4 x 10^4 cells per well. At 16-24 h after seeding, cells were transfected with 200 ng of pZFA constructs, 5 ng of pGL3-ZFA binding site reporters and 1 ng of pRL-CMV (Promega, Madison, WI, USA) using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. At 48 h after transfection, cells were washed once with Dulbecco's PBS (DPBS; Life Technologies, Carlsbad, CA, USA) and lysed with Passive Lysis Buffer (Promega, Madison, WI, USA). Luciferase expression was measured with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) using a Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA, USA) according to the manufacturer's instructions. Normalized luciferase activity was determined by dividing firefly luciferase activity by Renilla luciferase activity.

3.3.5. ZFA expression analysis

For expression analysis of designed zinc-finger activators, we seeded HEK293T cells onto a 24-well plate at a density of 2 x 10^4 cells per well. Twenty-four hours after seeding, cells were transfected with 500 ng of indicated ZFA expression constructs or pcDNA3.1 (Life Technologies, Carlsbad, CA, USA) backbone vector by Lipofectamine 3000 (Life Technologies, Carlsbad, USA), according to manufacturer instructions. At 48 h after transfection, cells were harvested and lysed with RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS) supplemented with EDTA-free Protease Inhibitor Cocktail Tablets (Roche, Basel, Switzerland). The Bio-Rad Protein

Assay Kit (Bio-Rad, Hercules, CA, USA) was used to determine protein concentration according to the manufacturer's instructions. Zinc-finger activator expression was analyzed by 4-12% SDS-PAGE (National Diagnostics, Atlanta, GA, USA). Samples were transferred onto a 0.2 μm nitrocellulose membrane as described [403] and detected with SuperSignal West Femto (Thermo Fischer, Waltham, MA, USA) and Amersham Hyperfilm ECL (GE Healthcare, Little Chalfont, UK) chemiluminescence film. Zinc-finger activators were detected by horseradish peroxidase-conjugated anti-HA monoclonal antibody (Clone 3F10; Roche, Basel, Switzerland). Vinculin was used as an internal control and detected using a mouse anti-Vinculin monoclonal antibody (Clone 7F9; Santa Cruz Biotechnology, Dallas, TX, USA) and horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Bio-Rad, Hercules, CA, USA).

3.3.6. J-Lat ZFA nucleofection

Episomal transfection of zinc-finger activators in J-Lat cells was performed by plasmid nucleofection as previously described [448]. Briefly, we seeded J-Lat cells onto a 10-cm dish at a density of 1 x 10^5 cells per mL. After 48 hours, 2 x 10^5 cells per transfection were centrifuged at 100 *g* for 10 min at room temperature and resuspended in Nucleofector Solution SE (Lonza, Basel, Switzerland) with 2 μg of indicated pcDNA ZF activator construct. Cells were transferred to 16-well Nucleocuvette (Lonza, Basel, Switzerland) and electroporated with by a 4D-Nucleofector System (Lonza, Basel, Switzerland) selecting the program CL-120. At 48 h after transfection, cells were washed twice with DPBS (Life Technologies, Carlsbad, CA, USA) and GFP expression was evaluated by flow cytometry (BD LSR II Flow Cytometer System; BD Biosciences, Franklin Lakes, NJ, USA). For each sample, 10,000 live events were collected, and data was analyzed using FlowJo (Tree Star, Inc., San Carlos, CA, USA).

3.3.7. CPP-ZFA protein expression and purification

Expression and purification of cell-penetrating zinc-finger activators was adapted from a previously optimized protocol [264]. Zinc-finger pET28b expression constructs were transformed into chemically competent *E. Coli* BL21(DE3) strain (Merck KGaA, Darmstadt, Germany). Overnight culture from a single colony was inoculated into 500 mL of LB media supplemented with 200 mM NaCl, 50 μg/ml kanamycin, 100 μM $ZnCl_2$ and 0.2% glucose. Cell culture was grown at 37 °C until OD_{600} reached 0.5 and then at room temperature until OD_{600} reached 0.8. Protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside

(IPTG; Thermo Fischer, Waltham, MA, USA) for 4 hours at room temperature. Cells were pelleted by centrifugation at 12,000 *g* for 5 min and stored at -20 °C until purification protocol.

Purification of Zinc-finger activators was carried by resuspending cell pellet in 20 mL of ZF binding buffer (20 mM HEPES pH 8.0, 2 M NaCl, 1 mM $MgCl_2$, 100 µM $ZnCl_2$ and 10% glycerol). This solution was supplemented with 1 mM β-mercaptoethanol, protease inhibitors (Roche, Basel, Switzerland) and 0.1% Triton X-100. Cells were lysed by sonication (10 min, 50% output, pulse on) and centrifuged at 12,000 *g* for 60 min at 4 °C. The supernatant was cleared by running through a 0.45 µM low protein binding filter. Cell lysate was transferred to a His GraviTrap flow gravity column (GE Healthcare, Little Chalfont, United Kingdom). Column was washed with 10 mL of 5 mM imidazole (Merck KGaA, Darmstadt, Germany) and with 5 mL of 35 mM imidazole in ZF binding buffer. ZF protein elution was performed with 300 mM imidazole in ZF binding buffer. Ten fractions of 0.5 mL eluted proteins were collected and supplemented with 100 mM L-Arginine. Fractions containing majority of eluted protein were selected, combined and buffer-exchanged to ZF storage buffer (20 mM HEPES pH 8.0, 500 mM NaCl, 1 mM $MgCl_2$, 100 µM $ZnCl_2$ and 10% glycerol and 100 mM L-Arg) using PD-10 desalting columns (GE Healthcare, Little Chalfont, United Kingdom). ZF proteins were then concentrated using an Amicon Ultra-15 Centrifugal Filter Unit (Merck KGaA, Darmstadt, Germany) and stored at -80 °C. Purified CPP-ZFA batches were analyzed by 4-12% SDS-PAGE (National Diagnostics, Atlanta, GA, USA) and stained using BlueSafe (NzyTech, Lisboa, Portugal).

3.3.8. CPP-ZFA binding analysis

Assessment of zinc-finger activator proteins binding to target sites was performed by enzyme-linked immunosorbent assay (ELISA). Briefly, ELISA plates were coated with 400 ng streptavidin per well overnight at 4 ºC. After rinsing with ddH_2O, wells were incubated with 25 ng biotin-marked oligonucleotides containing ZFA target site for one hour at 37 ºC. Plates were rinsed with ddH_2O and blocked with 3% BSA in ZNBA buffer (20 mM Tris-HCl pH 7.4, 90 mM KCl, 1 mM $MgCl_2$, 100 µM $ZnCl_2$) for one hour at 37 ºC. Serial dilutions of CPP-ZFA protein were prepared starting from 125 nM in ZF storage buffer supplemented with 1% BSA and 3 µg herring sperm DNA. Plates were incubated with protein dilutions for 2 hours at room temperature. Following washing with DPBS-Tween20 0.1%, wells were probed with anti-HA monoclonal antibody (Clone 3F10; Roche, Basel, Switzerland) in 1% BSA/ZF storage buffer for 30 min at room temperature. Following washing with ddH_2O, plates were developed with

ABTS (Calbiochem Merck KGaA, Darmstadt, Germany) conjugated with H_2O_2 for 15 minutes and analyzed by measuring Abs (405/492 nm) on Tecan Infinite M-200 plate reader.

3.3.9. J-Lat CPP-ZFA protein treatment

J-Lat cells were seeded 24 hours before protein treatment in 24-well plates with 2 x 10^5 cells per well. After 24 hours, cells were centrifuged at 300 *g* for 5 min at room temperature. Zinc-finger activators were diluted in supplemented RPMI medium at final concentration indicated. J-Lat cells were resuspended in zinc-finger protein solution and incubated at 37ºC and 5% CO_2 atmosphere for 90 minutes, unless otherwise indicated. After protein treatment, cells were washed twice with supplemented RPMI media and then moved to 37ºC and 5% CO_2 atmosphere. For each experiment, cells were also incubated with ZF storage buffer as mock control.

For analysis of HIV activation, cells were collected 48 hours after protein treatment, washed twice with DPBS (Life Technologies, Carlsbad, CA, USA) and GFP expression was evaluated by flow cytometry (BD LSR II Flow Cytometer System; BD Biosciences, Franklin Lakes, NJ, USA). For each sample, 10,000 live events were collected, and data was analyzed using FlowJo (Tree Star, Inc., San Carlos, CA, USA).

3.3.10. CPP-ZFA cell transduction analysis

Evaluation of CPP-ZFA cell entry by flow cytometry was performed through protein treatment of Jurkat cells with FITC-conjugated CPP-ZFA for 90 minutes. PBS1-VP64 and 3NLS-PBS1-VP64 were stained using the FluoReporter FITC Protein Labeling Kit (Thermo Fischer, Waltham, MA, USA), according to manufacturer's instructions. Jurkat cells were treated with increasing concentrations of PBS1-VP64 and 3NLS-PBS1-VP64 following the J-Lat CPP-ZFA protein treatment protocol described above in section 3.3.8. Cells were collected and washed twice with Trypsin 0.25%-EDTA (GE Healthcare, Little Chalfont, UK) and resuspended in DPBS (Life Technologies, Carlsbad, CA, USA). FITC positive cells were evaluated by flow cytometry (BD LSR II Flow Cytometer System; BD Biosciences, Franklin Lakes, NJ, USA). For each sample, 10,000 live events were collected, and data was analyzed using FlowJo (Tree Star, Inc., San Carlos, CA, USA).

Evaluation of 3NLS-PBS1-VP64 cell entry kinetics was assessed by western blot following protein treatment of J-Lat cells with 2 µM of purified protein, as described in section 3.3.8. Cells were collected at indicated timepoints, washed twice with Trypsin/EDTA (GE

Healthcare, Little Chalfont, UK) and lysed with RIPA buffer (25 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1 % SDS, pH 7.6) supplemented with EDTA-free Protease Inhibitor Cocktail Tablets (Roche, Basel, Switzerland). Protein quantification and 3NLS-PBS1-VP64 detection by western blot was performed as described above in section 3.3.4.

3.3.11. Statistical analysis

Statistical analyses for all experiments were performed from three independent experimental replicates ($n = 3$) unless otherwise indicated. Two-tailed Student's *t*-test was used for paired and unpaired samples (Prism Software 5.0, GraphPad Software).

3.4. RESULTS

3.4.1. Design of zinc-finger transcription factors targeting HIV-1 promoter

To reactivate latent HIV expression from viral reservoirs, we designed zinc-finger activators (ZFA) targeting the 5' long terminal repeat (5'LTR) promoter. Based on our previous experience with TALE activators [448], we concentrated on the "hot spot" region of the HIV promoter located from -200 bp from the transcription start site (TSS). We submitted this region to Zinc-finger Tools web server, an automated design tool to obtain the best zinc-finger domains from any given target sequence, based on a experimentally characterized zinc-finger database [451]. To enforce binding specificity, we selected zinc-finger matches mainly composed of GNN and ANN triplets, restricted at most to one CNN triplet **(Table 3.1)** [454]. We designed 4-finger (ZLT4A through ZLT4D) and 6-finger (ZLT6A and ZLT6B) domains to target HIV promoter **(Fig. 3.1A)**. While shorter 4-finger (12-bp targets) domains may improve binding activity [455], 6-finger (18-bp targets) domains confer genome-wide specificity of target [259].

Table 3.1 - Representation of zinc-fingers designed in this study to target HIV promoter.

ZF	Position[a]	Predicted target sequence (5'-3')	ZF α-Helix sequence[b]					
			F6	F5	F4	F3	F2	F1
ZLT4A	-163	GGC CCG AGA GCT			DPGHLVR	RNDTLTE	QLAHLRA	TSGELVR
ZLT4B	-134	AAG ACT GCT GAC			RKDNLKN	THLDLIR	TSGELVR	DPGNLVR
ZLT4C	-52	GTG GCG AGC CCT			RSDELVR	RSDDLVR	ERSHLRE	TKNSLTE
ZLT4D	-31	CAT ATA AGC AGC			TSGNLTE	QKSSLIA	ERSHLRE	ERSHLRE
ZLT6A	-146	CGG AGT ACT ACA AAG ACT	RSDKLTE	HRTTLTN	THLDLIR	SPADLTR	RKDNLKN	THLDLIR
ZLT6B	-140	ACT ACA AAG ACT GCT GAC	THLDLIR	SPADLTR	RKDNLKN	THLDLIR	TSGELVR	DPGNLVR

[a]Position relative to transcription start site of HIV-1 promoter.
[b]Aminoacids from position -1 to +6 relative to zinc-finger α-Helix are shown.

In addition, we explored previously designed zinc-fingers against HIV 5'LTR promoter (HLTR1, HLTR3 and HLTR6) or primer binding site (PBS) region (PBS1, PBS1a and PBS3) (**Fig. 3.1A; Table 3.2)**. These zinc-fingers demonstrated proof-of-principle of HIV inhibition when fused to a transcription repressor domain [344,346].

Table 3.2 - Representation of previously designed zinc-finger to target HIV promoter.

Zinc-fingers reported in [344,346].

ZF	Position[a]	Predicted target sequence (5'-3')	ZF α-Helix sequence[b]					
			F6	F5	F4	F3	F2	F1
HLTR1	+23	TGG GTG ACG AAT TCG GAG	RSDNLVR	TSGELVR	QSSNLAS	QSGDLRR	RSDVLVR	TSGHLVR
HLTR3	-80	GGA GGC GTG GCC TGG GCG	QSSHLVR	DPGHLVR	RSDVLVR	DCRDLAR	RSDHLTT	RSDDLVR
HLTR6	+118	ACA CTG AGA CCA TTG ATC	QRHSLTE	TSGSLVR	DKKDLTR	QLAHLRA	DPGALVR	SPADLTR
PBS1	+167	AAA TCT CTA GCA GTG GCG	QRANLRA	RGGWLQA	QRHSLTE	QSGDLRR	RSDVLVR	RSDDLVR
PBS1a[c]	+160	GTG TGG AAA atctcta GCA GTG GCG	RSDVLVR	RSDHLTT	QRANLRA	QSGDLRR	RSDVLVR	RSDDLVR
PBS3	+180	TGG CGC CCG AAC AGG GAC	RSDHLTT	HTGHLLE	RNDTLTE	DSGNLRV	RSDHLAE	DPGNLVR

[a]Position relative to transcription start site of HIV-1 promoter.
[b]Aminoacids from position -1 to +6 relative to zinc-finger DNA-recognition sequence are shown.
[c]PBS1a consists in two three-finger modules separated by a long flexible linker (Gly3SerGly4). The F6 to F4 modules bind the first half site (GTG TGG AAA) and the F3 to F1 modules bind the second half site (GCA GTG GCG). Nonbound DNA sequence is indicated by lowercase letters.

Each zinc-finger protein was fused to VP64 transactivation domain [254] that recruits the transcription machinery to induce gene expression [405,406]. ZFA constructs incorporate a C-terminal hemagglutinin (HA) tag for protein detection, and an internal nuclear localization signal (NLS) sequence between the DNA binding and transactivation domains **(Fig. 3.1B; Top)**. Western blot analysis of ZFA expression in HEK293T cells shows expression of all 4-finger and 6-finger ZFAs designed although with remarkable variance in protein levels among some constructs, possibly due to differences in protein conformational stability **(Fig. 3.1B; Bottom)**. The amino acid sequence of each protein is presented in **Table S1**.

We initially evaluated the potential of each ZFA to specifically induce gene expression, using a luciferase reporter assay previously described with TALE activators [398] that correlate luciferase expression with the ability of ZFA to recognize its target and stimulate gene activation. We constructed luciferase reporter plasmids for each ZFA that contained four direct repeats of each binding site to drive firefly luciferase expression **(Fig. 3.1C; Top)**. HEK293T cells were transfected with each ZFA construct and its corresponding reporter. We observed increased luciferase activation for all ZFA constructs compared to its reporter alone **(Fig. 3.1C; Bottom)**. From all designed ZFA, ZLT4D (~1,300-fold), HLTR6 (~1,600-fold), PBS1 (~400-fold) and PBS1a (~200-fold) expressively enhanced gene activation from their target sites. The

remaining ZFA constructs designed induced a modest ~3 to 30-fold gene activation over reporter background. Moderate levels of gene activation could only be explained partially by low levels of protein expression, as was the case of ZLT6B and HLTR1 activators. This result indicates that ZFA binding affinity towards its target site should play a major role in its potential for gene activation although we cannot exclude the contribution of the ZFA stability in the intracellular environment.

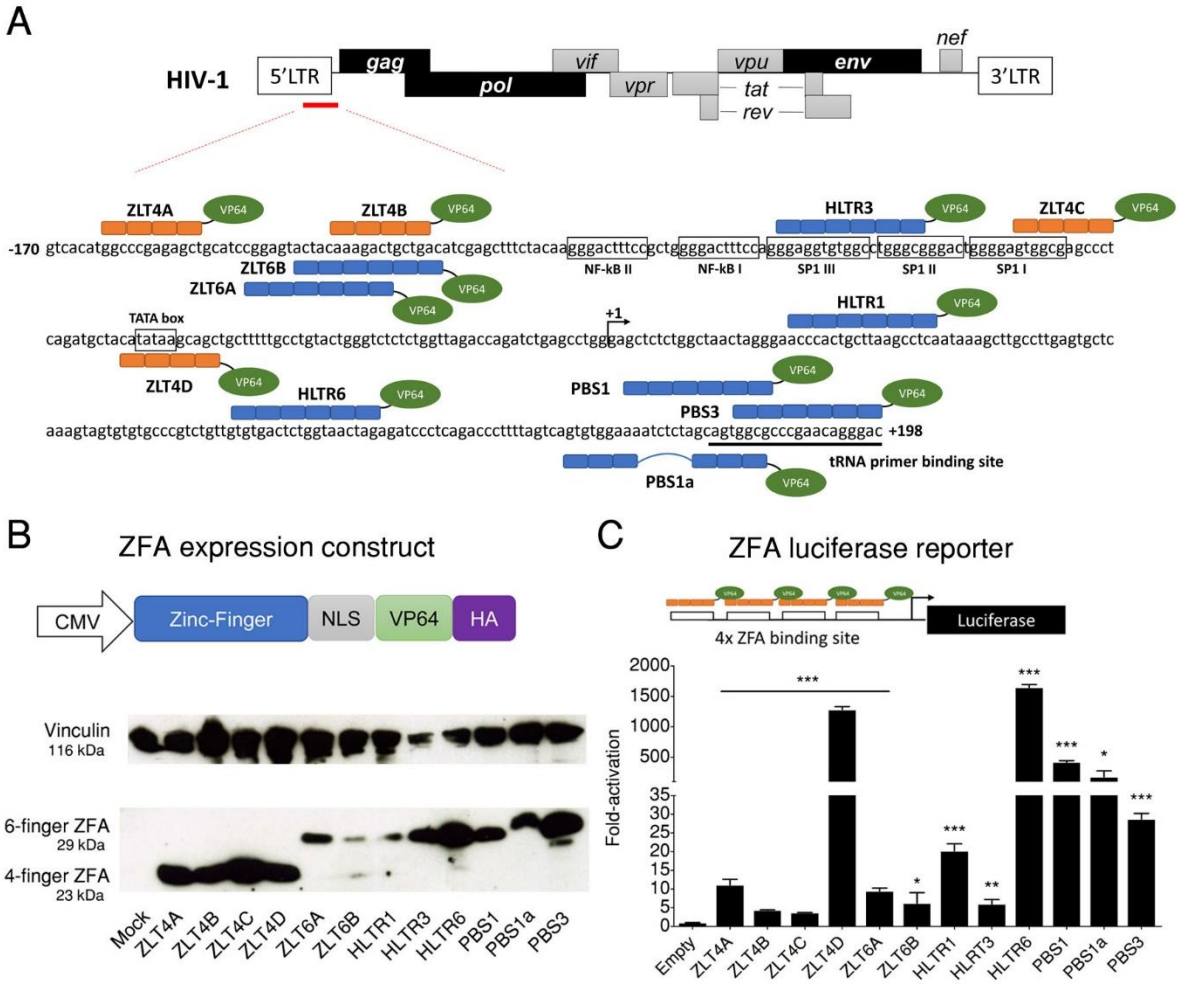


Figure 3.1– Zinc-finger activators (ZFA) designed to target HIV-1 promoter. (A) Schematic illustration of zinc-fingers target sites within 5' long terminal repeat (LTR) promoter and tRNA primer binding site of HIV-1. Most relevant endogenous transcription factor binding sites (NF-κB and SP1) are referenced. **(B) (Top)** Schematic representation of ZFA activator expression constructs. ZFA coding sequences are incorporated into a pcDNA backbone vector driven by the cytomegalovirus (CMV) promoter. VP64 denotes the tetrameric repeat of the herpes simplex virus VP16 transactivation domain, NLS stands for the nuclear localization signal derived from the simian virus (SV40) and HA indicates the hemagglutinin A tag. **(Bottom)** Western blot of 20 µg lysate of HEK293T transfected with indicated ZFA constructs. Samples were taken 48 h after transfection and probed with horseradish peroxidase-conjugated anti-HA and anti-Vinculin (loading control) antibodies. Mock indicates lysate from HEK293T cells transfected with empty pcDNA vector only. **(C) (Top)** Schematic representation of the luciferase reporter system containing four direct repeats of the zinc-finger target sites for each ZFA construct. **(Bottom)**

Fold-activation of luciferase expression after co-transfection of indicated ZFA with luciferase reporter plasmid into HEK293T cells. Luciferase expression was normalized to cells transfected with reporter plasmid only (Empty). *Renilla* luciferase expression vector was also co-transfected to normalize for transfection efficiency and cell number. Error bars indicate standard deviation of one experiment with three transfection replicates ($n = 3$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; *t*-test sample vs control (Empty)).

3.4.2. Zinc-finger activators specifically stimulate latent HIV-1 expression

In addition to its binding efficacy, the ability of a zinc-finger activator to induce gene expression is also connected to the location of its target sequence relative to the transcription start site. To evaluate the potential of ZFA to induce HIV transcription from the native promoter, we constructed a luciferase reporter driven by the full-length LTR and PBS regions, from -455 to +198 relative to TSS. This segment of HIV-1 contains all the essential elements that regulate viral gene expression and include all ZFA target sites. We co-transfected HEK293T cells with ZFA constructs and the LTR-PBS reporter (**Fig. 3.2A)**. As controls for specific target activation of 4-finger and 6-finger ZFA, respective non-targeting Aart4 (predicted target site: 5'-AGA AAA ACC AGG-3') and Aart6 (predicted target site: 5'-ATG TAG AGA AAA ACC AGG-3') ZFA were designed from the previously designed Aart zinc-finger [256], that in principle should not bind any sequence within the HIV provirus.

From 4-finger ZFA constructs, none could induce gene activation beyond non-targeting Aart4-VP64 control, which induces a 2-fold background luciferase activity, possibly due to non-specific activation caused by the presence of VP64 domain. The same effect was observed with 6-finger control Aart6-VP64. Within 6-finger ZFA constructs, HLTR6-VP64 (~3-fold) and PBS1-VP64 (~4-fold) were able to substantially increase LTR activation over the Aart6 control **(Fig. 3.2A)**, indicating these as the most potential zinc-finger activators to induce HIV-1 transcription. Efficient activation of LTR promoter by these ZFA also correlates with their ability to stimulate transcription from their target site observed in **Fig. 3.1C**. However, despite their strong ability to induce gene expression, ZLT4D and PBS1a activators were unable to induce LTR activation. Interestingly, HLTR3-VP64 activator repressed LTR background activity obtained from cells transfected with LTR-PBS reporter alone. This effect is due to HTLR3 target overlap with SP1 binding site, that outcompetes this endogenous factor and blocks LTR activation, highlighting the importance of zinc-finger location within the HIV promoter [344]. Combining different sets of ZFA did not caused a synergistic effect on LTR activation (data not shown), as previously demonstrated for TALE activators [448].

We set out to evaluate the potential of ZFA constructs to reactivate latent HIV-1 expression. For this purpose, latency reactivation was evaluated using the lymphocytic Jurkat-derived HIV latent cell line (J-Lat). This latency model harbors a full-length integrated HIV-1 proviral genome containing a GFP gene that serves as a reporter for viral gene expression (HIV1-ΔEnv-GFP) **(Fig. 3.2B)**. These latent cells do not express integrated provirus unless reactivated by a strong stimulus, such as one caused by tumor necrosis factor (TNF)-α [55]. J-Lat 10.6 clone, which is more sensitive to reactivating stimulus, was nucleofected with indicated HLTR6-VP64 or PBS1-VP64 constructs to evaluate HIV-1 expression after detection of GFP-positive cells by flow cytometry.

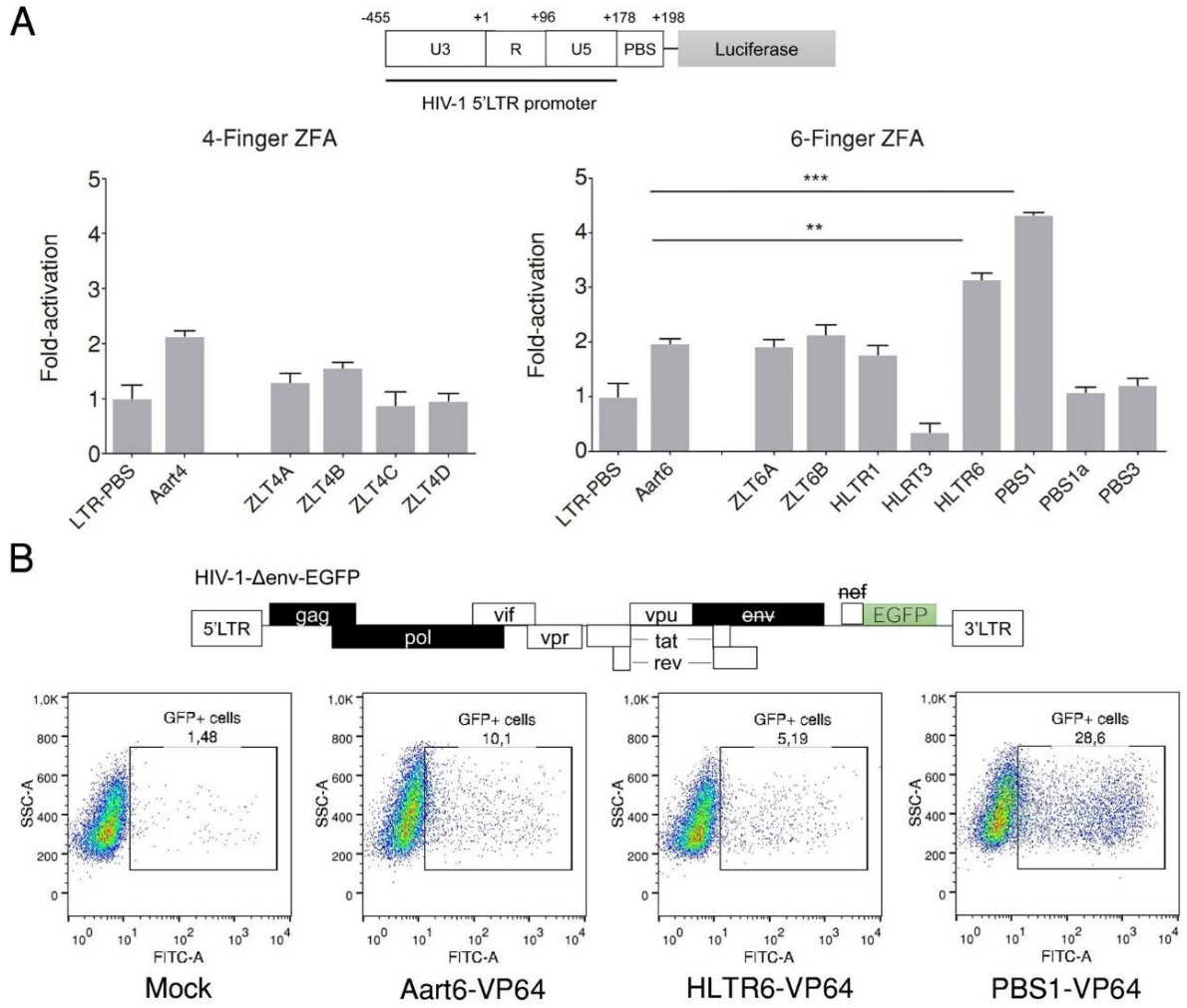


Figure 3.2– Zinc-finger activators target LTR promoter and activate latent HIV-1 expression. (A) (Top) Schematic representation of luciferase reporter used to evaluate ZFA activity from the HIV-1 LTR promoter. Full-length 5'LTR (U3-R-U5) and primer binding site (PBS) regions were placed upstream of firefly luciferase reporter. **(Bottom)** Fold-activation of luciferase expression in HEK293T cells co-transfected with indicated ZFA expression constructs and LTR-PBS luciferase reporter. Luciferase expression was normalized to that obtained with

transfection of reporter plasmid alone (LTR-PBS). *Renilla* luciferase expression vector was also co-transfected to normalize for transfection efficiency and cell number. Error bars indicate standard deviation of one experiment with three transfection replicates (n=3; $**p < 0.01$; $***p < 0.001$; *t*-test sample vs. control (Aart6)). **(B)** Percent of GFP-positive cells of J-Lat 10.6 cells nucleofected with LTR-targeted HLTR6-VP64 or PBS1-VP64, or non-specific Aart6-VP64 zinc-finger activators. "J-Lat" indicates non-transfected J-Lat 10.6 cells. GFP-positive cells were measured by flow cytometry at 48 hours after nucleofection. Dot plots are representative of a single experiment from three independent replicates.

Contrary to what observed with luciferase episomal assays in HEK293T, HLTR6-VP64 nucleofection lead to minor (~5% GFP-positive cells) reactivation of latent J-Lat 10.6 population, even lower to that observed with non-specific Aart6-VP64 control (~10%). However, PBS1-VP64 was able to significantly reactivate (~30% GFP-positive cells) latent population **(Fig. 3.2B)**, consistent with its ability to stimulate LTR transcription observed when co-transfected with luciferase reporter driven by HIV promoter **(Fig. 3.2A)**. Overall, these results indicate that PBS1-VP64 activator can specifically activate latent HIV expression from the LTR promoter. This zinc-finger targets the tRNA primer binding site region, being extremely conserved across distinct HIV subtypes, with full conservation in 80-90% of HIV-1 strains among subtypes of major (M) group **(Fig. S3.1)**, including the most dominant subtype B (prevalent in the Americas, Western Europe, Japan and Australia) and subtype C (prevalent in Southern and Eastern Africa, China and India). PBS1 zinc-finger activator was selected for subsequent studies on ZFA protein delivery to activate latent HIV infected populations.

3.4.3. Protein delivery of cell-penetrating zinc-finger activators reactivate latent HIV-1 expression

As a mean to explore their potential for direct protein delivery, we evaluated if zinc-finger activators could act as cell-penetrating peptides (CPP-ZFA) and activate latent HIV expression. We incorporated PBS1-VP64 activator into a pET28b vector for expression and purification of CPP-ZFA protein from *E. Coli* **(Fig. 3.3A)**. To enhance ZFA protein delivery, we fused three nuclear localization signal (NLS) repeats to the N-terminal region of PBS-VP64 activator. Incorporation of positively charged NLS sequences have previously shown to improve cell permeability of zinc-finger nucleases and enhance its gene editing activity [264]. SDS-PAGE analysis of purified CPP-ZFA **(Fig. 3.3B)** demonstrates the purity of these proteins.

To confirm that purified CPP-ZFA proteins retained their functionality, we performed an ELISA analysis to evaluate protein binding to DNA target site **(Fig. 3.3C)**. Both PBS1-VP64 and 3NLS-PBS1-VP64 activators similarly recognized PBS1 binding site in a concentration

dependent manner, indicating that addition of NLS repeats does not influence CPP-ZFA efficacy to bind DNA.

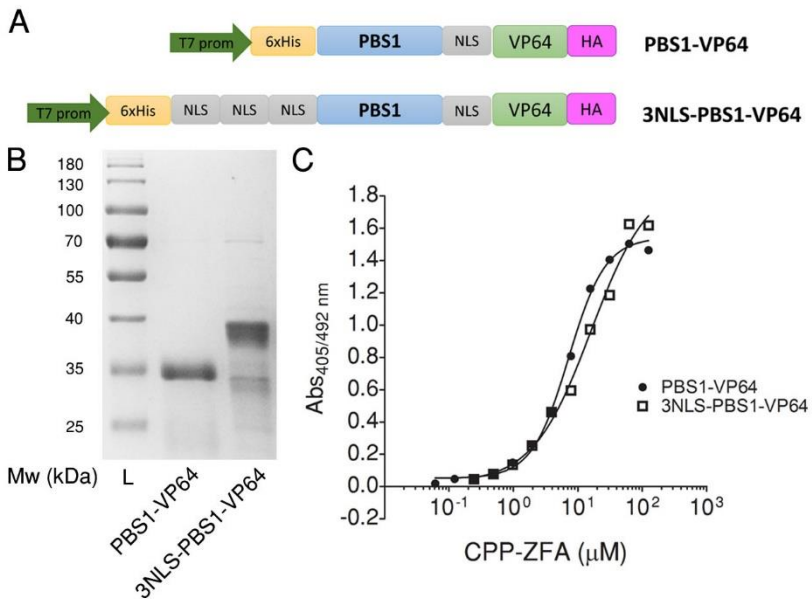


Figure 3.3– Expression and purification of cell-penetrating zinc-finger activators (CPP-ZFA) for protein delivery to HIV latent cells. (A) Schematic representation of CPP-ZFA protein expression vector for purification in *Escherichia Coli*. PBS1-VP64 was genetically fused to pET28b expression vector driven by IPTG-inducible T7 promoter. PBS1-VP64 contains a nuclear localization signal (NLS) between PBS1 zinc-finger and VP64 activation domains. 3NLS-PBS1-VP64 contains three additional NLS repeats at N-terminal of DNA-binding domain to enhance cell permeability. 6xHis indicates six histidine tag for protein purification. HA indicates the hemagglutinin A tag for protein detection. **(B)** SDS-PAGE of 0.1 nmol of CPP-ZFA proteins produced in *E. Coli* BL21(DE3). CPP-ZFA were detected using BlueSafe protein staining. **(C)** ELISA analysis of PBS1-VP64 and 3NLS-PBS1-VP64 target binding.

We next determined whether CPP-ZFA could penetrate HIV latent cells and activate viral expression. PBS1-VP64 and 3NLS-PBS1-VP64 proteins were incubated with the J-Lat 10.6 latency model described above. Strong reactivation of J-Lat 10.6 cells was observed following treatment with 3NLS-PBS1-VP64 in a concentration-dependent manner, reaching a maximum of ~40% GFP-positive cells after treatment with 8 µM **(Fig. 3.4A)**. Opposed to 3NLS-PBS1-VP64, we only detected significant reactivation of J-Lat cells treated with PBS1-VP64 (~15% GFP-positive cells) at maximum protein concentration (8 µM), indicating the importance of NLS repeats to promote CPP-ZFA protein delivery and activation of latent HIV cells. Comparison of cell-entry between PBS1-VP64 and 3NLS-PBS1-VP64 through detection of FITC-conjugated CPP-ZFA in Jurkat T lymphocytes confirms that incorporation of 3NLS sequence is necessary to promote efficient cell entry **(Fig. 3.4B; Left)**, reaching approximately 70% cell transfection with 2 µM protein treatment **(Fig. 3.4B; Right)**. Analysis of live J-Lat 10.6 population following treatment with 3NLS-PBS1-VP64 indicated that cell viability is

drastically reduced after treatment with more than 2 µM of CPP-ZFA **(Fig. 3.4C)**. Therefore, subsequent studies were performed with 2 µM protein treatment.

Defining optimized parameters for protein delivery could be crucial to further enhance activation of latent HIV by CPP-ZFA. Increasing CPP-ZFA time of incubation with J-Lat cells clearly improved activation of latent population, reaching a maximum of ~40% HIV activation with 3NLS-PBS1-VP64 treatment for 6 hours **(Fig. 3.3D)**. Supplementing cell medium with $ZnCl_2$ or L-arginine, as previously reported for cell-penetrating zinc-finger nucleases [264], did not enhanced CPP-ZFA activity (data not shown). To further observe CPP-ZFA entry and function kinetics within J-Lat 10.6 cells, we incubated these cells with 3NLS-PBS1-VP64 for 6 hours and monitored CPP-ZFA cell entry by western blot and stimulation of latent HIV expression through flow cytometry, over a period of 5 days **(Fig. 3.3E)**. We observed that efficient entry 3NLS-PBS1-VP64 is only detected after 3 hours, reaching a maximum between 9 and 18 hours, following degradation to almost complete extinction by 72 hours. In turn, HIV stimulation occurs at 12 hours and persists for up to 72 hours at which CPP-ZFA is already degraded, after it declines possibly due to the superior fitness of HIV latent over HIV expressing cells. This data indicates that protein delivery of CPP-ZFA remains in the latent cells for a short period but sufficient to sustain HIV activation.

Multiple short-term treatments with CPP-ZFA further increased the fraction of reactivated population **(Fig. 3.3F)**, considerably improving HIV activation over extending CPP-ZFA incubation to 6 hours **(Fig. 3.3C)**. For all conditions tested, we did not observe any significant changes in J-Lat 10.6 reactivation when treated with non-specific 3NLS-Aart6-VP64, clearly indicating that viral gene expression is specifically induced by HIV-targeting PBS1 activator. In conclusion, these results indicate that short-term CPP-ZFA protein delivery is able to penetrate latent cells and specifically activate HIV expression from the LTR promoter.

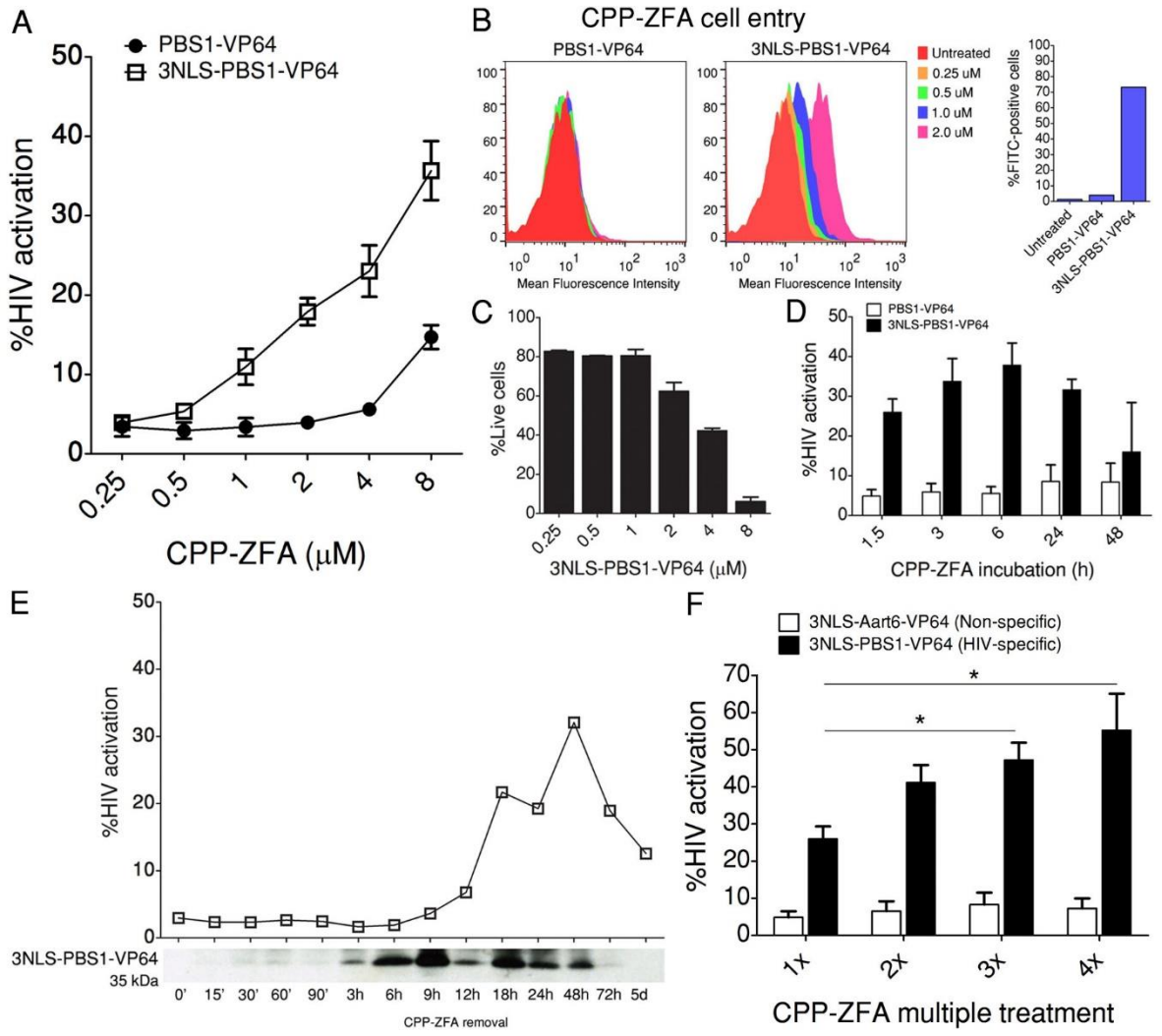


Figure 3.4- Protein delivery of CPP-ZFA induce latent HIV expression. (A) Percentage of HIV activation in J-Lat 10.6 population following treatment with 0.25 µM to 8 µM of PBS1-VP64 (filled circles) or 3NLS-PBS1-VP64 (open squares) proteins for 90 minutes. HIV activation of J-Lat 10.6 population was evaluated through detection of GFP-positive cells by flow cytometry at 48 hours after protein incubation. **(B) (Left)** Mean fluorescence intensity analysis determined by flow cytometry of Jurkat cells treated with increasing concentrations (from 0.25 µM to 2 µM) of FITC-stained PBS1-VP64 or 3NLS-PBS1-VP64 for 90 minutes. **(Right)** Percentage of FITC-positive cells of Jurkat cells untreated or treated with 2 µM of FITC-stained PBS1-VP64 or 3NLS-PBS1-VP64 for 90 minutes. **(C)** Percentage of live cells of J-Lat 10.6 cells treated with increasing concentrations (from 0.25 µM to 8 µM) of 3NLS-PBS1-VP64 for 90 minutes. Cell viability were assessed through flow cytometry at 48 hours after protein treatment by gating the forward-scatter/side-scatter (FSC/SSC) live population. Percentage of live cells is normalized for untreated J-Lat cells. **(D)** Percentage of HIV activation in J-Lat 10.6 population treated with 2 µM of PBS1-VP64 (white columns) or 3NLS-PBS1-VP64 (black columns) for different periods of protein incubation. HIV activation of J-Lat 10.6 population was evaluated through detection of GFP-positive cells by flow cytometry at 48 hours after protein incubation. **(E)** Flow cytometry analysis of HIV activation (Top) and western blot (20 µg lysate) analysis of CPP-ZFA entry (Bottom) of J-Lat 10.6 cells treated with 2 µM of 3NLS-PBS1 for 6 hours. Samples were collected for analysis at the indicated timepoints. **(F)** Percentage of HIV activation in J-Lat 10.6 population following multiple treatments with 2 µM of 3NLS-Aart6-VP64 (white columns) or 3NLS-

PBS1-VP64 (black columns) proteins. Cells were incubated with CPP-ZFA protein for 1.5h per treatment. HIV activation of J-Lat 10.6 population was evaluated through detection of GFP-positive cells by flow cytometry at 48 hours after protein incubation. Error bars indicate standard deviation of three independent experiments (n=3; $*p < 0.05$).

3.5. DISCUSSION

The "shock and kill" strategy has gained consensus as the most promising approach to eliminate HIV reservoirs. However, non-targeted therapeutics developed to activate viral expression were unable to reduce the reservoir size and associated with toxic effects generally caused by uncontrollable host immune response [29,52,78,390]. The emergence of genome engineering tools have greatly expanded the possibilities to target the HIV genome and promote an antiviral effect [456]. Site-directed nucleases and recombinases are able to recognize and excise the integrated HIV proviral genome from infected cells [372–374,383], nevertheless genotoxicity caused by unwanted cuts at the human genome [240] or emergence of resistant HIV strains generated by double-strand breaks at the viral genome [377–381] raise concerns about the safety of this approach.

Opposite to nucleases, engineered activators have been shown to modulate gene expression [254,269,303] without causing disruptive DNA breaks and generally with absence of off-target effects [396]. The elevated number of recent studies [429–432,434–436,448,449] reporting the design of synthetic activators to induce latent HIV expression clearly demonstrate the potential of this approach to target HIV reservoirs. However, drawbacks associated with standard delivery methods, including toxicity, integration-derived mutagenesis or low efficiency in some cell-types, have limited application of these cutting-edge tools into patients [457]. Zinc-fingers possess the innate ability to penetrate the anionic cell membrane, owing to the presence of positively charged residues within its backbone [450,458], retaining both cell-transduction and DNA-binding functions in the same molecule. Accordingly, we explored its potential to open new perspectives for protein delivery of engineered activators to target HIV reservoirs.

In this study, we report the construction of a cell-penetrating zinc-finger activator (CPP-ZFA) for direct protein delivery and activation of latent HIV infected cells. We designed zinc-finger activators (ZFA) to target the HIV 5'LTR promoter and induce viral gene expression. Contrary to that observed with TALE activators [448], ZFA are less consistent in activating gene expression. PBS1-VP64 was identified as the single ZFA to strongly activate latent HIV expression, reaching levels comparable to those reported in our study using TALE activators

[448]. Despite HLTR6-VP64 ability to activate gene expression from the LTR promoter, this zinc-finger might not access its target site within chromatin [344], as evidenced by its inability to stimulate latent HIV expression in J-Lat 10.6 model. PBS1 target site overlaps tRNA primer binding site and flanking sequences [346] located at the 3' end of the LTR promoter unbound to nucleosome, therefore accessible to binding of engineered activators [459]. More importantly, this region is described as the most conserved segment of HIV-1 genome [346,460], being required for initiation of viral retro-transcription following binding of human $tRNA^{Lys}$ [461]. Although gene activation is generally stronger when engineered transcription factors are targeted upstream of TSS, these can be also efficient from downstream regions [241], as demonstrated by PBS1-VP64 capacity to stimulate HIV transcription.

We further demonstrated that PBS1-VP64 activator can be directly delivered to latent cells as cell-penetrating peptide. Significant stimulation of latent HIV expression was only obtained with the inclusion of multiple nuclear localization sequences (NLS) at the N-terminal region of PBS1-VP64. Despite efficient gene knockout previously demonstrated through cell-penetrating zinc-finger nucleases [263,264], transactivation domains such as VP64 are characterized by the presence of acidic and negatively charged residues [462], which might challenge its translocation through the anionic cell membrane. In fact, PBS1-VP64 activator alone presents neutral charge at physiological pH (estimation includes the presence of polyhistidine-tag, which further increase net charge). Incorporation of 3NLS repeats confer high positive charge to this CPP-ZFA (+15.2), demonstrated by its superior cell transduction and activity. This result correlates with previous report by Liu *et al.* that demonstrates the potential of NLS peptides to increase overall positive net charge and enhance protein delivery of zinc-finger nucleases [264].

Protein delivery of CPP-ZFA improves temporal control of engineered activators function over gene delivery methods. These "hit and run" strategies are suitable for latent HIV activation, for which engineered activators remain in the cell shortly enough to stimulate HIV transcription but without causing potentially harmful off-target gene modulation from prolonged exposure. In fact, we observed that short-term presence of CPP-ZFA within latent HIV cells is able to sustain viral expression. Controlling the protein amount, time of incubation or multiple protein injections can better adjust the optimal conditions to enhance gene activation.

Despite TALE or CRISPR/Cas9 exponential growth for therapeutic applications [240], these technologies do not match zinc-fingers protein delivery potential due to its supercharged surface and relative small size [287]. In addition, the human origin of zinc-finger domains might

potentially reduce risks of immunogenicity. Other studies have shown efficient cell-penetration of ZFA by conjugating other protein-transduction domains [463,464], leaving room to further optimize cell-penetrating capacity of zinc-finger activators. One of these studies went as far as to demonstrate *in vivo* localization of CPP-ZFA in the brain, demonstrating its capacity to cross blood-brain barrier and modulate gene expression [464], a desirable feature to target potential HIV reservoirs in the central nervous system [465]. Still, translation of this technology into infected patients might require conjugation of engineered activators with ligands that target these into the relevant cell populations, particularly resting CD4 T lymphocytes. Targeted delivery of zinc-finger nucleases have shown to enhance cell transduction efficacy [466], further supporting the potential of zinc-finger protein delivery for *in vivo* applications.

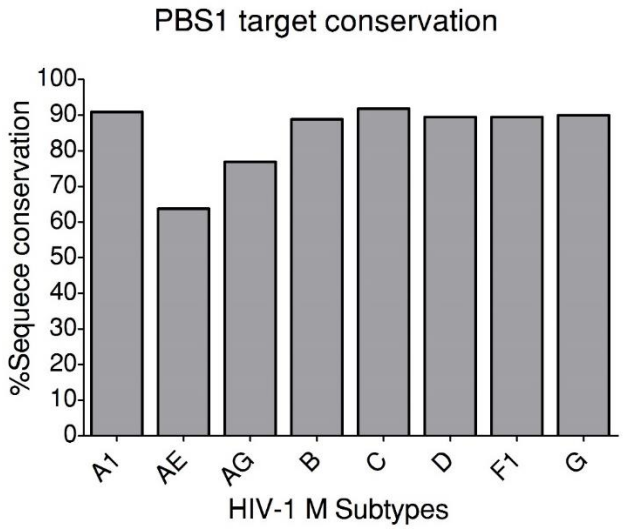
In conclusion, we provide evidence that cell-penetrating zinc-finger activators can be directly delivered to latent HIV cells without any carrier and strongly stimulate viral expression. This study is an important step to approach novel "shock and kill" strategies to eradicate latent HIV reservoirs.

3.6. ACKNOWLEDGMENTS

We thank Catarina Santos for collaboration and assistance in the zinc-finger protein production and experimental assays. We thank Thomas Gaj and Jia Liu for advice in the Zinc-finger design and protein production. The following reagents were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, and NIH: pNL4-3 from Dr. Malcolm Martin, Jurkat clone E6-1 from Dr. Arthur Wess and J-Lat Full Length clones from Dr. Eric Verdin. This work was supported by grants from Alto Comissariado para a Saúde and Fundação para a Ciência e Tecnologia- Ministério da Educação e Ciência (FCT-MEC) (VIH/SAU/0013/2011, VIH/SAU/0020/2011 and HIVERA/0002/2013), The Skaggs Institute for Chemical Biology and the National Institutes of Health (DP1CA174426).

3.7. SUPPLEMENTARY INFORMATION

3.7.1. Supplementary Figures



Supplementary Figure S 3.1- Sequence conservation of the PBS1 zinc-finger binding site. Percentage of HIV Major group (M) subtype strains with complete homology to PBS1 target site. Data based on 2016 edition of the HIV Sequence Database (http://hiv-web.lanl.gov).

3.7.2. Supplementary Tables

Supplementary Table S 3.1- Zinc-finger activator protein sequences used in this study. ZF α-Helix DNA-binding domain is colored orange. Nuclear localization signal (NLS) sequence is highlighted grey. VP64 domain is colored green. HA tag is colored purple.

>ZLT4A-VP64
MAQAALEPGEKP
YKCPECGKSFSTSGELVRHQRTHTGEKP
YKCPECGKSFSQLAHLRAHQRTHTGEKP
YKCPECGKSFSRNDTLTEHQRTHTGEKP
YKCPECGKSFSDPGHLVRHQRTHTGKKTSGQAGQASPKKKRKVGRADALDDFDLDMLGSDALDDFDLDMLGSDALDDFDLD
MLGSDALDDFDLDMLINYPYDVPDYAS

>ZLT4B-VP64
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YKCPECGKSFSTSGELVRHQRTHTGEKP
YKCPECGKSFSTHLDLIRHQRTHTGEKP
YKCPECGKSFSRKDNLKNHQRTHTGKKTSGQAGQASPKKKRKVGRADALDDFDLDMLGSDALDDFDLDMLGSDALDDFDLD
MLGSDALDDFDLDMLINYPYDVPDYAS

>ZLT4C-VP64
MAQAALEPGEKP
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YKCPECGKSFSRSDDLVRHQRTHTGEKP
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MLGSDALDDFDLDMLINYPYDVPDYAS

>ZLT4D-VP64
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MLGSDALDDFDLDMLINYPYDVPDYAS

>ZLT6A-VP64
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>ZLT6B-VP64
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>HLTR1-VP64
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YKCPECGKSFSQSGDLRRHQRTHTGEKP
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YKCPECGKSFSTSGELVRHQRTHTGEKP
YKCPECGKSFSRSDNLVRHQRTHTGKKTSGQAGQASPKKKRKVGRADALDDFDLDMLGSDALDDFDLDMLGSDALDDFDLD
MLGSDALDDFDLDMLINYPYDVPDYAS

>HLTR3-VP64
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>HLTR6-VP64
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>PBS1-VP64
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MLGSDALDDFDLDMLINYPYDVPDYAS

>PBS1a-VP64
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YKCPECGKSFSQSGDLRRHQRTHTG
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>PBS3-VP64
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>Aart4-VP64
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YKCPECGKSFSQRANLRAHQRTHTGEKP
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>Aart6-VP64
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MLGSDALDDFDLDMLINYPYDVPDYAS

Supplementary Table S 3.2- Cell-penetrating zinc-finger activator protein sequences used in this study. Histidine tag is colored orange. ZF DNA-binding domain is colored blue. Nuclear localization signal (NLS) sequence is highlighted grey. VP64 domain is colored green. HA tag is colored purple.

>PBS1-VP64
MGSSHHHHHHSSGLVPRGSHMLEAALEPGEKPYACPECGKSFSRSDDLVRHQRTHTGEKPYKCPECGKSFSRSDVLVRHQRTH
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HTGEKPYKCPECGKSFSQRANLRAHQRTHTGKKTSGQAGQASPKKKRKVGRADALDDFDLDMLGSDALDDFDLDMLGSDAL
DDFDLDMLGSDALDDFDLDMLINYPYDVPDYAS

>3NLS-PBS1-VP64
MGSSHHHHHHSSGLVPRGSHMPKKKRKVLDPKKKRKVPGMAPKKKRKVGIHGVPAALEPGEKPYACPECGKSFSRSDDLVRH
QRTHTGEKPYKCPECGKSFSRSDVLVRHQRTHTGEKPYKCPECGKSFSQSGDLRRHQRTHTGEKPYACPECGKSFSQRHSLTEH
QRTHTGEKPYKCPECGKSFSRGGWLQAHQCTHTGEKPYKCPECGKSFSQRANLRAHQRTHTGKKTSGQAGQASPKKKRKVGR
ADALDDFDLDMLGSDALDDFDLDMLGSDALDDFDLDMLGSDALDDFDLDMLINYPYDVPDYAS

>3NLS-Aart6-VP64
MGSSHHHHHHSSGLVPRGSHMPKKKRKVLDPKKKRKVPGMAPKKKRKVGIHGVPAALEPGEKPYACPECGKSFSRSDHLAEH
QRTHTGEKPYKCPECGKSFSDKKDLTRHQRTHTGEKPYKCPECGKSFSQRANLRAHQRTHTGEKPYACPECGKSFSQLAHLRAH
QRTHTGEKPYKCPECGKSFSREDNLHTHQRTHTGEKPYKCPECGKSFSRRDALNVHQRTHTGKKTSGQAGQASPKKKRKVGRA
DALDDFDLDMLGSDALDDFDLDMLGSDALDDFDLDMLGSDALDDFDLDMLINYPYDVPDYAS

Supplementary Table S 3.3- Primer sequences for the construction of the luciferase reporter plasmids used in this study. Zinc-finger binding sites are underlined. Restriction sites are in bold.

>5' ZF-Fwd
GGGAATTC**CATATG**CTCGAGGCCGCCATGGCCCAGGCGGCC

>5' 3NLS-ZF-Fwd
ACGC**GTCGAC**CCAAAGAAGAAACGGAAAGTACCCGGGATGGCCCCCAAGAAAAAGCGGAAAGTG
GGCATCCACGGCGTGCCTGCCGCCCTCGAGCCCGGGGAGAAG

>3' VP64-Rev
C**GAGCTC**TCAAGAAGCGTAGTCCGGAACG

>5' ZF-Luc-ZLT4A
ACTGCTAT**CTCGAG**GGCCCGAGAGCTTAGCGGGCCCGAGAGCTTAGCGGGCCCGAGAGCTTAGCG
GGCCCGAGAGCTTAGCGATCTGCGATCTAAGTAAGCT

>5' ZF-Luc-ZLT4B
ACTGCTAT**CTCGAG**AAGACTGCTGACTAGCGAAGACTGCTGACTAGCGAAGACTGCTGACTAGCG
AAGACTGCTGACTAGCGATCTGCGATCTAAGTAAGCT

>5' ZF-Luc-ZLT4C
ACTGCTAT**CTCGAG**GTGGCGAGCCCTTAGCGGTGGCGAGCCCTTAGCGGTGGCGAGCCCTTAGCG
GTGGCGAGCCCTTAGCGATCTGCGATCTAAGTAAGCT

>5' ZF-Luc-ZLT4D
ACTGCTAT**CTCGAG**CATATAAGCAGCTAGCGCATATAAGCAGCTAGCGCATATAAGCAGCTAGCG
CATATAAGCAGCTAGCGATCTGCGATCTAAGTAAGCT

>5' ZF-Luc-ZLT6A
ACTGCTAT**CTCGAG**CGGAGTACTACAAAGACTTAGCGCGGAGTACTACAAAGACTTAGCGCGGAG
TACTACAAAGACTTAGCGCGGAGTACTACAAAGACTTAGCGATCTGCGATCTAAGTAAGCT

>5' ZF-Luc-ZLT6B
ACTGCTAT**CTCGAG**ACTACAAAGACTGCTGACTAGCGACTACAAAGACTGCTGACTAGCGACTAC
AAAGACTGCTGACTAGCGACTACAAAGACTGCTGACTAGCGATCTGCGATCTAAGTAAGCT

>5' ZF-Luc-HLTR1
ACTGCTAT**CTCGAG**TGGGTGACGAATTCGGAGTAGCGTGGGTGACGAATTCGGAGTAGCGTGGGT
GACGAATTCGGAGTAGCGTGGGTGACGAATTCGGAGTAGCGATCTGCGATCTAAGTAAGCT

>5' TALE-Luc-HLTR3
ACTGCTAT**CTCGAG**GGAGGCGTGGCCTGGGCGTAGCGGGAGGCGTGGCCTGGGCGTAGCGGGAGG
CGTGGCCTGGGCGTAGCGGGAGGCGTGGCCTGGGCGTAGCGATCTGCGATCTAAGTAAGCT

>5' ZF-Luc-HLTR6
ACTGCTAT**CTCGAG**ACACTGAGACCATTGATCTAGCGACACTGAGACCATTGATCTAGCGACACTG
AGACCATTGATCTAGCGACACTGAGACCATTGATCTAGCGATCTGCGATCTAAGTAAGCT

>5' ZF-Luc-PBS1
ACTGCTAT**CTCGAG**AAATCTCTAGCAGTGGCGTAGCGAAATCTCTAGCAGTGGCGTAGCGAAATCT
CTAGCAGTGGCGTAGCGAAATCTCTAGCAGTGGCGTAGCGATCTGCGATCTAAGTAAGCT

>5' ZF-Luc-PBS1a
ACTGCTAT**CTCGAG**GTGTGGAAAatctctaGCAGTGGCGTAGCGGTGTGGAAAatctctaGCAGTGGCGTA
GCGGTGTGGAAAatctctaGCAGTGGCGTAGCGGTGTGGAAAatctctaGCAGTGGCGTAGCGATCTGCGA
TCTAAGTAAGCT

>5' TALE-Luc-PBS3
ACTGCTAT**CTCGAG**TGGCGCCCGAACAGGGACTAGCGTGGCGCCCGAACAGGGACTAGCGTGGCG
CCCGAACAGGGACTAGCGTGGCGCCCGAACAGGGACTAGCGATCTGCGATCTAAGTAAGCT

>3' Luc-Rev
CGTTTTCCCGGTACCAGAT

>5' LTR-PBS-Fwd
CG**ACGCGT**TGGAAGGGCTAATTTGGTCCCA

>3' LTR-PBS-Rev
CTA**GCTAGC**GTCCCTGTTCGGGCGCC

CHAPTER IV

General discussion and future perspectives

Latent non-replicative HIV reservoirs evade retroviral inhibitors but continue to source novel infection events, being considered one of the main obstacles to complete eradication. For this reason, reactivating the latent expression is considered critical to expose these cells to elimination through virus-mediated cytopathic effects or host immune response, providing a "shock and kill" effect on HIV reservoirs [467]. Pharmacological strategies that aim to stimulate HIV-1 latent cells by modulating cell signaling or epigenetic marks associated with the HIV provirus were unsuccessful so far. HDAC inhibitors remain the single latency reversal agent to be tested in clinical studies to disrupt HIV reservoirs. Vorinostat [87,88], panobinostat [468] and romidepsin [469] induced HIV-1 RNA transcription from aviremic ART-treated patients, however these failed to reduce the HIV reservoir size. Such decrease is vital to control the HIV infection by the host immune system in the absence of ART and attain a "functional cure". Low levels of HIV stimulation and the heterogeneity of processes or cell subsets associated with latent HIV are major hurdles that might limit the effect of these agents [467]. More importantly, the non-selective mode of action of pharmacological drugs may raise severe side effects by triggering an uncontrolled immune response [29]. The emergence of gene therapy field has provided novel targets and expanded possible ways to counter HIV infection [160]. Still, most strategies are unable to target HIV reservoirs, masked due to the absence of viral expression or cellular markers. In fact, the presence of an integrated silenced provirus within the cell chromatin represents the only consistent trait that can distinguish an HIV latently infected from a healthy cell.

In this dissertation, we presented novel gene-targeted strategies to specifically stimulate HIV latent expression and induce elimination of viral reservoirs. We set out to explore the potential of genome engineering tools to generate synthetic activators that could specifically stimulate latent HIV expression without compromising the normal cell function**.** This approach also proposes to generate a broad effect by stimulating HIV reservoirs in a context independent manner, regardless of the provirus integrated position [470] or availability of endogenous transcription factors [471]. To complement this approach, we also engineered an HIV-responsive suicidal lentivector to drive expression of a toxic gene and enforce elimination of stimulated HIV reservoirs.

In Chapter I, we presented a general introduction to the HIV pathogenesis, describing the establishment of latent infections and its critical importance to obstruct HIV eradication by current antiretroviral treatment. State-of-the-art of gene therapy methods with focus on genome engineering approaches to target and modulate gene expression are depicted as a promising

alternative to pharmacological treatments. We present gene therapeutic and genome engineering strategies to target HIV infection with focus on unmet needs and drawbacks of available treatment scheme.

In Chapter II, we describe the design of synthetic activators based on TALE domains to target conserved regions of HIV 5'LTR promoter and stimulate transcription of latently infected cells [448]. In the pursuit for antiviral strategies, the versatility of programmable DNA-binding platforms confers a considerable advantage over available pharmacological or peptide-mediated inhibitors that are directed to viral proteins. Their straightforward DNA recognition mode facilitates the generation of site-directed genome engineering effectors that target conserved regions within integrated HIV provirus. Additionally, these proteins can be engineered and virtually re-directed to any HIV subtype or potentially emergent resistant strain, simply required by knowledge of the viral genomic sequence. Related to this, TALE proteins possess unique attributes particularly valuable for targeted anti-HIV strategies. TALEs are highly flexible proteins that adapt to target sequence variations commonly associated with the diversity of circulating HIV strains. More specifically, the existence of degenerated RVD domains that recognize multiple or every bp [244,245] facilitates adjustment to a specific target prone for single nucleotide polymorphism at a specific position. In addition, TALEs can be engineered to tolerate frameshift deletions mutations through conformational arrangements [472]. In comparison to other programmable DNA-binding proteins, TALEs are frequently associated with increased specificity and less off-target events [473]. Comparison between these platforms for gene activation is limited, regardless TALE activators have shown to outperform ZF or CRISPR in most circumstances [306,308,396,474].

We generated TALE activators that mapped the HIV 5'LTR promoter to track the region more prone for activation of viral transcription. Four TALE activators (TLT5, TLT6, TLT7 and TLT8) caused strong activation of the LTR promoter, targeting a small region identified as a "hotspot" to stimulate viral transcription due to the presence of endogenous *cis*-regulatory sites that should cooperate with synthetic TALE activators. This finding also correlates with other studies reporting synthetic activators to reactivate HIV latency [430,434,436,449,475]. We demonstrate that combination of selected TALE activators (TLT5-8) positioned in proximity can promote a synergistic upregulation of HIV transcription in a similar manner to what occurs with natural gene regulators. TLT5-8 strongly reactivated latent HIV population in the widely-used J-Lat 10.6 cell line model, still its activity was limited in more suppressed latent clones, indicating that the level of repression can affect their performance. Nevertheless, we could

alleviate suppression of latent HIV provirus within such unfavorable contexts by combining these artificial transcription factors with chromatin-remodeling HDAC inhibitors.

Class-selective HDAC inhibitors, particularly those targeting HDAC isotype 3 [476], cooperated with TLT5-8 and enhanced stimulation of repressive J-Lat clones. Further studies are required to investigate if the influence of HDAC inhibitors is to facilitate TALE activators access to the HIV promoter [459], increase TALE transgene expression [477] and/or release additional blocks to viral transcription by indirectly stimulating productive elongation [412,413]. The complex epigenetic regulation involving HDAC enzymes inevitably determine that the broad effect of HDAC inhibitor drugs affects expression of numerous host genes [478]. To further reduce potential off-target gene regulation caused by HDAC inhibitors, conjugating TALE activators with HIV site-directed epigenetic modulators [241] could represent an attractive concept to further potentiate the specificity of this approach. Programmable acetyltransferases [276] directed to HIV provirus have already shown to modulate a positive effect on latent expression [434]. Furthermore, testing novel activation domains [312,314] that recruit complementary transcription factors should increase potency of synthetic activators. Nonetheless, given the multifactorial nature of HIV-1 latency [27], this study further supports that combinatorial strategies [479] are more likely to expand the range of biologically diverse reservoirs that can be addressed.

Future studies should address the delivery method of these programmable synthetic activators with potential for *in vivo* application. Transient delivery through IDLV is highly desirable given the lentivirus efficacy to transduce both active and resting T lymphocytes [140]. Nevertheless, undesirable recombination owing to the presence of repetitive elements within TALE backbone poses an obstacle to implement this system [290]. Codon-optimized TALEs [277,480] to minimize repetitive domains and RT-inactive lentivirus for direct mRNA delivery [441] are alternative options to overcome this bottleneck. Other alternative viral delivery systems should also be considered for this approach. Opposed to TALEN-mediated gene editing that requires dimerization of site-directed nucleases, gene modulation can be performed by single TALE transcription factor which enable packaging into small AAV vectors [275]. On the other hand, the large packaging capacity of adenoviral vectors would enable gene delivery of multiple TALEs from a single viral particle [291]. TALE-mediated reactivation of HIV latency through such delivery platforms would then be more relevant to evaluate its potential in cells extracted from aviremic ART-treated patients [481], or primary cell [419,482] and

animal [483] models of HIV latency that recapitulate the biological properties of HIV reservoirs that occur *in vivo*.

Recent reports have raised concerns that stimulating HIV reservoirs is not sufficient to lead their elimination. The ineffective CTL-mediated immune response of HIV patients [91], along with the emergence of CTL-resistant strains [92] indicate that supplementary approaches might be required to clear the reactivated population. Additionally, the elimination of stimulated HIV reservoirs must be swift enough to prevent viral spread to surrounding tissues. Conjugating ART with latency reversal agents does not guarantee prevention dissemination, as low levels of replication in tissues under suboptimal ART drug concentration contributes to the residual viremia observed in treated patients [484,485]. Here we also presented a suicide gene therapy approach to directly clear latent HIV cells through conjugation of designed TALE activators with an HIV-responsive suicide lentivector. Lentiviral vectors previously developed to express suicide genes (e.g. toxins) conditioned to the presence of Tat and Rev showed promising results to specifically eliminate HIV infected cells [189,190,486]. Despite its specificity, these therapeutic vectors are unable to tackle transcriptionally silent HIV reservoirs that do not express these regulatory viral proteins.

We aimed to expand this approach for targeting HIV reservoirs by taking advantage of the gene-targeted modulation of designed TALE activators. We engineered a Tat/Rev-dependent lentiviral vector [188] with a modified 5'LTR promoter that does not contain binding sites for these synthetic activators. By doing so, we could conjugate both technologies and generate a "shock and kill" effect on HIV reservoirs without depending on the host immune response. In association with TALE activators, a low dose of HIV-responsive suicidal lentivector driving expression of Diphtheria toxin specifically induced moderate cell death of HIV latent cells with minimal effect on uninfected cells. Despite the potential of HIV suicide gene therapy, this approach faces safety issues that might impair its applicability in infected patients and requires further improvements. Leaking of suicide gene expression from excessive transcription activity may trigger death of healthy cells, requiring moderate HIV-dependent expression levels but sufficient to induce cell apoptosis in HIV infected cells. Consequently, the choice of suicidal gene for our lentivector is critical to maximize its efficacy and safety. The high potency of pathogenic bacterial toxins circumvents the requirement of high levels of expression to mediate cell apoptosis [425]. For instance, one molecule of diphtheria toxin is sufficient to trigger cell death [487], therefore representing a suitable option for this system. Moreover, the generation of resistant cells [488] allows production of viral particles

incorporating this suicide gene. Other alternative candidates such as human pro-apoptotic [486,489,490] or prodrug-activating [421,491] suicide genes can be tested for increased safety. Further engineering of lentiviral vector through incorporation of HIV cis-inhibitory sequences from the *gag* gene [486] could also help to minimize HIV-independent leaky expression. Additionally, transient delivery of this system as non-integrative lentivirus [190] should further enhance its safety. Future studies might be addressed to incorporate an HIV-independent TALE activator cassette into the suicide lentivector and generate a "all-in-one" therapeutic vector that could maximize efficacy of this "shock and kill" strategy.

Although this study provides gene-targeted methods designed to eliminate HIV latent cells, significant challenges will exist in clinical translation of these approaches. Gene-delivery systems to HIV infected cells have limited efficacy and specificity. However, advances in targeted viral delivery promises to overcome this bottleneck [159]. Recent reports showed that lentivirus and AAV can be designed with CD4 [492,493] or CCR5 [494] targeting specificity, enabling efficient transduction of HIV-susceptible CD4+ T lymphocytes or macrophages both *in vitro* and *in vivo*. The great potential of AAV for *in vivo* genome engineering [495] has already shown promising results to remove the HIV provirus within infected tissues of animal models [496,497] Targeting HIV reservoirs however face problematic obstacles due to their presence in hardly accessible niche organs and most importantly the absence of cellular markers [29]. Identification of potential biomarkers associated with HIV latent infections in CD4 T cells [498] may open new perspectives for delivery of gene therapeutic drugs directly to the HIV reservoir.

In Chapter III, we described a "gene-free" approach to stimulate HIV latent expression through direct protein delivery of cell-penetrating zinc-finger activators (CPP-ZFA). For this purpose, we took advantage of the high cell-transduction potential of Cys2-His2 zinc-finger domains, owed to the high frequency of positively charged lysine and arginine residues present within its backbone [450]. This approach combines the cell-penetrating ability with site-specific targeting into a single molecule, which may overcome limitations associated with viral or non-viral gene delivery methods [499], i.e. immunogenicity/toxicity and efficiency. In addition, direct protein delivery enables short-term exposure of genome engineering platforms, previously correlated with reduction of off-target effects in comparison with long-term expression from nucleic acids [287]. Recent findings of *in vivo* gene modulation by cell-penetrating zinc-fingers [464] further demonstrate the relevance of this platform to target HIV reservoirs in infected patients.

We designed and screened zinc-finger activators (ZFA) that could target the 5'LTR promoter and stimulate HIV transcription. Opposed to TALE activators described in Chapter II, ZFA are far less consistent in activating gene expression, potentially explained by the context-dependent variance in protein affinity and specificity between the selected target site and corresponding zinc-finger module [243,500]. We narrowed all potential ZFA to PBS1-VP64, a zinc-finger that targets the HIV primer binding site, one of the most conserved regions among HIV subtypes [460]. PBS1-VP64 strongly activated latent HIV expression from the J-Lat 10.6 model, albeit it binds downstream of TSS.

The potential of PBS1 as CPP-ZFA was evidenced by its strong efficacy to stimulate latent HIV expression through protein delivery, even surpassing in some conditions (longer protein exposure, multiple treatments) the efficiency observed from transgene expression. The activation mediated by short-term protein delivery could be sufficient to trigger elimination of HIV reservoirs while avoiding off-target gene modulation from prolonged exposure to engineered zinc-fingers [263]. The presence of multiple positively charge NLS was required to promote entry of sufficient CPP-ZFA amounts to activate HIV expression. The cationic properties of CPP is of critical importance for interaction and internalization through the anionic lipid bilayer [501]. Despite the positive charge involving PBS1, coupling of this zinc-finger to VP64 acidic domain decreased overall net charge. However, incorporation of 3NLS repeats conferred high positive charge to this CPP-ZFA at physiological pH, demonstrated by its superior cell transduction and activity. Still, the elevated concentration required to efficiently stimulate latent HIV reactivation notes the necessity for further improvements of CPP-ZFA cell transduction efficiency and/or gene activation potency to translate this approach *in vivo*. The former might be attained by further increasing net charge by addition of extra NLS repeats, or other positively charged CPP [501]. Multiple protein treatments should also reduce the required dosage, facilitated by the human origin of zinc-fingers that reduce possibility of humoral immune responses. Gene activation enhancement could be attained by testing novel and more potent activation or epigenetic effectors that were reported to outperform VP64 in many applications [241]. More importantly, CPP-ZFA must be targeted to HIV latent cells, mostly composed of resting CD4 T lymphocytes [42]. Cell-penetrating peptides lacks cell-type specificity but can be directed at target populations through varied assembly strategies to targeting domains [502]. This approach was already reported to enhance protein delivery of zinc-finger nucleases [466], and therefore should be translated to CPP-ZFA targeted activation of HIV reservoirs.

CHAPTER V

Concluding Remarks

In conclusion, the present thesis provides novel gene-targeted methods to mediate elimination of HIV latently infected cells. We demonstrate the potential of TALE-based engineered activators to recognize the HIV promoter and specifically stimulate latent expression. Furthermore, the epigenetic modulation caused by small-molecule HDAC inhibitors can synergistically potentiate TALE-mediated stimulation of HIV latency, providing a novel insight for combinatorial strategies to eradicate HIV reservoirs. On the other hand, we explored an engineered suicidal lentivector to mediate cell death of HIV latent cells in association with TALE activators, enforcing direct elimination of HIV reservoirs while preventing spreading of novel viral infections. Finally, we show that cell-penetrating zinc-finger activators can be directly delivered to HIV latent cells and induce viral expression, providing the basis for a novel delivery method to target these reservoirs. Overall, our study demonstrates the potential of programmable synthetic activators and suicide lentivectors to tackle HIV reservoirs, paving the way for the next generation of therapeutic molecules for improved treatment of HIV/AIDS pandemic.

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